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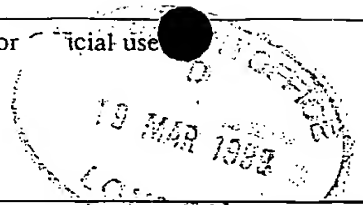

22MAR99 E434268-1 D02029
P01/7700 0.00 - 9906437.0

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Request for grant of a Patent

Form 1/77 **Patents Act 1977**

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1 Please give the title of the invention VACCINE

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4. Agent's or applicant's reference number *(if applicable)* MJWD/B45175

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❽ Checklist

8a Please fill in the number of sheets for each of the following types of document contained in this application

Continuation sheets for this Patents Form 1/77 [–]

Claim(s) 3 Description 26

Abstract [ ] Drawing(s) [ ]

8b Which of the following documents also accompanies the application?

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Priority documents *(please state how many)* [–]

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Patents Form 7/77 - Statement of Inventorship and Right to Grant [–]

Patents Form 9/77 - Preliminary Examination Report [–]

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MARCUS J W DALTON
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VACCINES

The present invention relates to polysaccharide antigen – Protein D conjugates, their use as immunogenic compositions, their manufacture and the use of such conjugates in medicines.

Polysaccharide antigen based vaccines are well known in the art, and four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3,4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33. .

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet these vaccines have not been licensed for use in children less than two years of age because they are poorly immunogenic in this age group.

The licensed polysaccharide vaccines listed above have different demonstrated clinical efficacy. The Vi polysaccharide vaccine has been estimated to have an efficacy between 55% and 77% in preventing culture confirmed typhoid fever (Plotkin and Cam, Arch Intern Med 155: 2293-99). The meningococcal C polysaccharide vaccine was shown to have an efficacy of 79% under epidemic conditions (De Wals P, et al. Bull World Health Organ. 74: 407-411). The 23-valent pneumococcal vaccine has shown a wide variation in clinical efficacy, from 0% to 81% (Fedson et al. Arch Intern Med. 154: 2531-2535). The efficacy appears to be related to the risk group that is being immunised, such as the elderly, Hodgkin's disease, splenectomy, sickle cell disease and agammaglobulinemics (Fine et al Arch Intern Med. 154:2666-2677), and also to the disease manifestation. Pneumococcal pneumonia and Otitis media are diseases, which do not have demonstrated protection by the 23-valent vaccine. It is generally accepted that the protective efficacy of the pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component

polysaccharide (Ed. Williams et al. New York Academy of Sciences 1995 pp. 241-249).

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies which have been designed to overcome this lack of immunogenicity include the linking of the polysaccharide to large highly immunogenic protein carriers, which provide bystander T-cell help.

Examples of these highly immunogenic carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD). A number of problems are associated with each of these commonly used carriers, including both problems in production of GMP constructs and also in immunological characteristics of the constructs. The present invention provides a new carrier for use in the preparation of peptide-based immunogen constructs, that does not suffer from the aforementioned disadvantages.

However, despite the common use of these carriers and their success in the induction of anti polysaccharide antibody responses they are associated with several drawbacks. For example, it is known that antigen specific immune responses may be suppressed by the presence of preexisting antibodies directed against the carrier, in this case Tetanus toxin (Di John *et al*; Lancet, December 16, 1989). In the population at large, a very high percentage of people will have pre-existing immunity to both DT and TT as people are routinely vaccinated with these antigens. In the UK for example 95% of children receive the DTP vaccine comprising both DT and TT. Other authors have described the problem of epitope suppression to peptide vaccines in animal models (Sad *et al*, Immunology, 1991; 74:223-227; Schutze *et al*, J. Immunol. 135: 4, 1985; 2319-2322).

In addition, for vaccine which require regular boosting the use of highly immunogenic carriers such as TT and DT are likely to suppress the polysaccharide antibody response after several injections. These multiple vaccinations may also be accompanied by undesirable reactions such as delayed type hyperresponsiveness (DTH).

KLH is known as potent immunogen and has already been used as a carrier for IgE peptides in human clinical trials. However, some adverse reactions (DTH-like reactions or IgE sensitisation) as well as antibody responses against antibody have been observed.

The selection of a carrier protein, therefore, for a polysaccharide based vaccine will require a balance between the necessity to use a carrier working in all patients (broad MHC recognition) and the induction of high levels of anti-polysaccharide antibody responses and of low antibody response against the carrier.

The carriers used previously for polysaccharide based vaccines, therefore have many disadvantages.

The present invention provides a protein D from *Haemophilus influenzae*, or fragments thereof, as a carrier for polysaccharide based immunogenic composition, including vaccines. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragment will preferably contain the N-terminal 1/3 of the protein.

Protein D is an IgD–binding protein from *Haemophilus influenzae* (EP 0 594 610 B1) and is a potential immunogen. The inventior, Forsgren, suggests that it may be fused to other genes and expressed to form fusion proteins.

Polysaccharides to be conjugated to Protein D contemplated by the present invention include, but are not limited to the Vi polysaccharide antigen against *Salmonella typhi*, meningococcal polysaccharides including type A, C, W135 and Y, the polysaccharide and modified polysaccharides of group B meningococcus, polysaccharides from *Staphylococcus aureus*, polysaccharides from *Streptococcus agalactae* and *streptococcus pneumoniae* polysaccharides from Mycobacterium, e.g. *Mycobacterium tuberculosis,* such as mannophosphoinisitides trehaloses, mycolic acid, mannose capped arabinomannans, the capsule therefrom and arabinogalactans, polysaccharide from *Cryptococcus neoformans*, the lipopolysaccharides of non-typeable *Haemophilus influenzae*, the capsular polysaccharide from *Haemophilus influenzae b*, the lipopolysaccharides of *Moraxella catharralis*, the lipopolysaccharides of *Shigella sonnei*, the lipopeptidophosphoglycan (LPPG) of *Trypanosoma cruzi*, the cancer associated gangliosides GD3, GD2, the tumor

associated mucins, especially the T-F antigen, and the sialyl T-F antigen, and the HIV associated polysaccharide that is structurally related to the T-F antigen.

The polysaccharide may be linked to the carrier protein by any known method. In particular by CDAP conjugation.

The cyanylating reagent 1-cyano-dimethylaminopyridinium tetrafluoroborate (CDAP) is preferably used for the synthesis of polysaccharide-protein conjugates. CDAP is a water soluble reagent and coupling a higher electrophilic cyano group than CNBr which may also be used in compiling the polysaccharide to the protein. The cyanilation reaction can be performed under relatively mild conditions and avoid hydrolysis of alkaline sensitive polysaccharides. This synthesis allows direct coupling to a carrier protein.

The polysaccharide is solubilized in water or a saline solution. CDAP is dissolved in acetonitrile and added immediately to the polysaccharide solution. The CDAP reacts with the hydroxyl groups of the polysaccharide to form cyanate ester. After the activation step, the carrier protein is added. Amino groups of lysine react with the activated polysaccharide to form an isourea covalent link.

After the coupling reaction, a large excess of glycine is then added to quench residual activated functions. The product is then passed through a gel permeation to remove unreacted carrier protein and residual reagents. Accordingly the invention provides a method of producing polysaccharide protein D conjugates comprising the steps of activating the polysaccharide and linking the polysaccharide to the protein D.

In a preferred embodiment of the invention there is provided a vaccine formulation for the prevention of *Streptococcus pneumoniae* infections.

Streptococcus pneumoniae is a gram positive bacteria that is pathogenic for humans, causing invasive diseases such as pneumonia, bacteremia and meningitis, and diseases associated with colonisation, such as acute Otitis media. The mechanisms by which pneumococci spread to the lung, the cerebrospinal fluid and the blood is poorly understood. Growth of bacteria reaching normal lung alveoli is inhibited by their relative dryness and by the phagocytic activity of alveolar macrophages. Any anatomic or physiological changes of these co-ordinated defences tend to augment the susceptibility of the lungs to infection. The cell-wall of

Streptococcus pneumoniae has an important role in generating an inflammatory response in the alveoli of the lung (Gillespie et al , I&I 65: 3936).

Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise protein D polysaccharide conjugates, wherein the polysaccharide are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 11 serotypes are included in the vaccine, for example the vaccine in one embodiment includes the protein D – capsular polysaccharide conjugate wherein the polysaccharide are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are included. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent vaccine above. For peadiatric vaccination (where Otitis media is of more concern), it is preferred to include polysaccharide conjugates from serotypes 6A and 19A to the 11 valent vaccines mentioned above.

In a further embodiment of the invention there is provided a Neisseria Meningitidis vaccine; in particular from serotypes A, B, C W-135 and Y. Neisseria Meningitidis is one of the most important causes of bacterial meningitis. The carbohydrate capsule of these organisms can act as a virulence determinant and a target for protective antibody. Carbohydrates are nevertheless well known to be poor immunogens in young children. Their poor immunogenicity is though to be due to the T-cell independent nature of polysaccharide antigens. The absence of a T-cell response prevents B-cell proliferation and maturation, as well as the induction of an immunological memory. The present invention overcomes these limitations by covalently coupling of capsular polysaccharides to protein D carrier which contains T-cell epitopes.

In an alternative embodiment of the invention there is provided a Protein D capsular polysaccharide of *Haemophilus influenzae b* (PRP) conjugate.

The present invention also contemplates combination vaccines which provide protection against a range of different pathogens.

A preferred combination includes a vaccine that affords protection against Neisseria Meningitidis A and C infection wherein the polysaccharide antigen from at least one, and preferably both A and C serotypes are linked to protein D.

Haemophilus influenza polysaccharide based vaccine may be formulated with the above combination vaccines.

Many Paediatric vaccines are now given as a combination vaccine so as to reduce the number of injections a child has to receive. Thus for Paediatric vaccines other antigens may be formulated with the vaccines of the invention. For example the vaccines of the invention can be formulated with, or administered separately, but at the same time with the well known 'trivalent' combination vaccine comprising Diphtheria toxoid (DT), tetanus toxoid (TT), and pertussis components [typically detoxified Pertussis toxoid (PT) and filamentous haemagglutinin (FHA) with optional pertactin (PRN) and/or agglutinin 1+2], for example the marketed vaccine INFANRIX-DTPa™ (SmithKlineBeecham Biologicals) which contains DT, TT, PT, FHA and PRN antigens, or with a whole cell pertussis component for example as marketed by SmithKlineBeecham Biologicals s.a., as Tritanrix™. The combined vaccine may also comprise other antigen, such as Hepatitis B surface antigen (HBsAg), Polio virus antigens (for instance inactivated trivalent polio virus - IPV), *Moraxella catarrhalis* outer membrane proteins, non-typeable *Haemophilus influenzae* proteins, *N.meningitidis* B outer membrane proteins.
Examples of preferred *Moraxella catarrhalis* protein antigens include: OMP106 WO 97/41731 (Antex) & WO 96/34960 (PMC), OMP21, LbpA & LbpB WO 98/55606 (PMC),TbpA & TbpB WO 97/13785 & WO 97/32980 (PMC), CopB Helminen ME, et al (1993) Infect. Immun. 61:2003-2010. UspA1/2 WO93/03761 (University of Texas), and OmpCD. Examples of non-typeable *Haemophilus influenzae* antigens include Fimbrin protein, (US 5766608 - Ohio State Research Foundation) and Fusions comprising peptides therefrom (eg LB1 Fusion) (US 5843464 - Ohio State Research Foundation),OMP26
WO 97/01638 (Cortecs) , P6 EP 281673 (State University of New York), TbpA and TbpB, Hia, Hmw1,2,Hap, and D15.

Preferred Peadiatric vaccines contemplated by the present invention are:

- *N.meningitidis* C polysaccharide – conjugate and *Haemophilus influenzae b* conjugate, optional with *N.meningitidis* A polysaccharide conjugate, provided that at least one polysaccharide antigen, and preferably all are conjugated to protein D.
- *N.meningitidis* C polysaccharide protein D conjugate *Haemophilius influenzae b* PRP conjugate, DT, TT, Pertussis component Hepatitis B surface antigen and I.P.V.
- *Streptococcus pneumoniae* protein D – polysaccharide antigen and one or more antigens from *Moraxella catarrhalis* and/or non-typeable *Haemophilia's influenzae*.

For the prevention of pneumoniae in the elderly (+55 years) population and Otitis media in Infants, it is a preferred embodiment of the invention to combine a multivalent streptococcus pneumonia polysaccharide – protein D antigens as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination, include but are not limited to, pneumolysin Nucleic Acids Res 1990 Jul 11;18(13):4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2." Mitchell TJ, Mendez F, Paton JC, Andrew PW, Boulnois GJ , Biochim Biophys Acta 1989 Jan 23;1007(1):67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ. WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al) -.WO 99/03884 (NAVA), PspA and transmembrane deletion variants thereof US 5804193 (Briles et al), PspC (WO 97/09994 - Briles et al). PsaA (Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*." Berry AM, Paton JC). Streptococcal choline binding protein (WO97/41151); Glyceraldehyde –3- phosphate – dehydrogenase (I&I 64: 3544), HSP 70 (WO96/40928).

Accordingly the present invention provides an immunogenic composition comprising a *Streptococcus pneumoniae* – polysaccharide – protein D conjugate and a *Streptococcus pneumoniae* protein antigen.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The polysaccharide – protein D conjugate antigens of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

For Paediatric uses, it is preferred to adjuvant the vaccine with an aluminium salt phosphate. For elderly vaccine it is preferred that the adjuvant be selected to be a preferably aluminium preferential inducer of a TH1 type of response.

High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to he antigen.

It is important to remember that the distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of Il-4, IL-5, IL-6, IL-10. Suitable adjuvant systems include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt .

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, o a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also a preferential inducer of a TH1 response and are suitable for use in the present invention.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial

vaccination, subjects may receive one or several booster immunisations adequately spaced.

EXAMPLE I

Neisseria Meningitidis C polysaccharide – Protein D conjugate (PSC-PD)

A: EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

Starting materials

The Protein D encoding DNA

Protein D is highly conserved among H. influenzae of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) I & I *59* : 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322, in which bacteriophage λderived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman et al., 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The E. coli strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, λ Kil - cI857 ΔH1). N99 and SA500 are E. coli K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilizes signals from λ phage DNA to drive the transcription and translation of inserted foreign genes. The vector

contains the λ promotor PL, operator OL and two utilization sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross et al., 1985). Vectors containing the PL promotor, are introduced into an E. coli lysogenic host to stabilize the plasmid DNA. Lysogenic host strains contain replication-defective λ phage DNA integrated into the genome (Shatzman et al., 1983). The chromosomal λ phage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promotor and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic E. coli strain used for the production of the protein D carrier is a derivative of the standard NIH E. coli K12 strain N99 (F - su - galK2, lacZ - thr -). It contains a defective lysogenic λ phage (galE::TN10, λ Kil - cI857 ΔH1). The Kil –phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the λ phage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, λ Kil - cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson et al. 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino

acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37°C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant E. coli strain is referred to as ECD4.

Expression of protein D is under the control of the λ P_L promotor/ O_L Operator. The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from λ P_L at low temperature by binding to O_L. Once the temperature is elevated cI is released from O_L and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D is described below:

The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final OD_{650} = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

B: MANUFACTURE OF POLYSACCHARIDE C

The source of group C polysaccharide is the strain C11 of N. meningitidis. This is fermented using classical fermentation techniques (EP 72513). The dry powder polysaccharides used in the conjugation process are identical to Mencevax (SB Biologicals s.a.).

Solid pre-cultures

An aliquot of C11 strain is thawed and 0.1ml of suspension is streaked on one Mueller Hinton medium petri dish supplemented with yeast extract dialysate (10%, v/v) and incubated for 23 to 25hrs at 36°C in a water saturated air incubator.

The surface growth is then re-suspended in sterilized fermentation medium and inoculated with this suspension on one Roux bottle containing Mueller Hinton

medium supplemented with yeast extract dialysate (10%, v/v) and sterile glass beads. After incubation of the Roux bottle during 23 to 25hrs at 36°C in a water saturated air incubator, the surface growth is re-suspended in 10ml sterile fermentation medium and 0.2 to 0.3ml of this suspension are inoculated onto 12 other Mueller Hinton medium Roux bottles.

After incubation during 23 to 25hrs at 36°C in a water saturated air incubator, surface growth is re-suspended in 10ml sterile fermentation medium. The bacterial suspension is pooled in a conical flask.

This suspension is then aseptically transferred into the fermenter using sterile syringes.

Fermentation

The fermentation of meningococcus is performed in fermenters contained in a clean room under negative pressure. The fermentation is generally completed after 10-12hrs corresponding to approximately 10^{10} bacteria/ml (i.e. the early stationary phase) and detected by pH increase.

At this stage, the entire broth is heat inactivated (12 min at 56°C) before centrifugation. Before and after inactivation, a sample of the broth is taken and streaked onto Mueller Hinton medium petri dishes.

C: PS PURIFICATION

The purification process is a multi-step procedure performed on the entire fermentation broth. In the first stage of purification, the inactivated culture is clarified by centrifugation and the supernatant is recovered.

Polysaccharides purification is based on precipitation with a quaternary ammonium salt (Cetyltrimethylammonium Bromide/CTAB,CETAVLON R). CTAB forms insoluble complexes with polyanions such as polysaccharides, nucleic acid and

proteins depending on their pI. Following ionic controlled conditions, this method can be used to precipitate impurities (low conductivity) or polysaccharides (high conductivity).

The polysaccharides included in clarified supernatant are precipitated using a diatomaceous earth (CELITE^R 545) as matrix to avoid formation of insoluble inert mass during the different precipitations/purifications.

Purification scheme for N. meningitidis polysaccharide C:

Step1: PSC-CTAB complex fixation on CELITE^R 545 and removal of cells debris, nucleic acids and proteins by washing with CTAB 0.05%.

Step 2: Elution of PS with EtOH 50%. The first fractions which are turbid and contain impurities and LPS are discarded. The presence of PS in the following fractions is verified by floculation test.

Step3: PS-CTAB complex re-fixation on CELITE R 545 and removal of smaller nucleic acids and proteins by CTAB 0.05% washing.

Step 4: Elution of PS with EtOH 50%. The first turbid fractions are discarded. The presence of PS in the following fractions is verified by floculation test.

The eluate is filtered and the filtrate containing crude polysaccharide collected.

The polysaccharide is precipitated from the filtrate by adding ethanol to a final concentration of 80 %.

The polysaccharide is then recovered as a white powder, vaccum dried and stored at -20°C.

D: CDAP CONJUGATION

Conjugation of PSC and PD

For conjugation of PSC and PD, the CDAP conjugation technology was preferred to the classical CNBr activation and coupling via a spacer to the carrier protein. The polysaccharide is first activated by cyanylation with 1-cyano-4-dimethylamino-

pyridinium tetrafluoroborate (CDAP). CDAP is a water soluble cyanylating reagent in which the electrophilicity of the cyano group is increased over that of CNBr, permitting the cyanylation reaction to be performed under relatively mild conditions. After activation, the polysaccharide can be directly coupled to the carrier protein through its amino groups without introducing any spacer molecule. The unreacted estercyanate groups are quenched by means of extensive reaction with glycine. The total number of steps involved in the preparation of conjugate vaccines is reduced and most importantly potentially immunogenic spacer molecules are not present in the final product.

Activation of polysaccharides with CDAP introduces a cyanate group in the polysaccharides and dimethylaminopyridine (DMAP) is liberated. The cyanate group reacts with NH2-groups in the protein during the subsequent coupling procedure and is converted to a carbamate.

PSC activation and PSC-PD coupling

Activation and coupling are performed at +25°C.

120 mg of PS is dissolved for at least 4h in WFI.

CDAP solution (100 mg/ml freshly prepared in acetonitrile) is added to reach a CDAP/PS (w/w) ratio of 0.75.

After 1 min 30, the pH is raised up to activation pH (pH 10) by addition of triethylamine and is stabilised up to PD addition.

At time 3 min 30, NaCl is added to a final concentration of 2M.

At time 4 min, purified PD is added to reach a PD/PS ratio of 1.5/1; pH is immediately adjusted to coupling pH (pH 10). The solution is left for 1h under pH regulation.

Quenching

6 ml of a 2M glycine solution is added to the PS/PD/CDAP mixture. The pH is adjusted to the quenching pH (pH 8.8). The solution is stirred for 30 min at the working T°, then overnight at +2-8°C with continuous slow stirring.

PS-PD purification

After filtration (5 µm), the PS-PD conjugate is purified in a cold room by gel permeation chromatography on a S400HR Sephacryl gel to remove small molecules (including DMAP) and unconjugated PD:

- Elution: NaCl 150 mM pH 6.5.
- Monitoring: UV 280 nm, pH and conductivity.

Based on the different molecular size of the reaction components, PS-PD conjugates are eluted first followed by free PD and finally DMAP. Fractions containing conjugate as detected by DMAB (PS) and µBCA (protein) are pooled. The pooled fractions are sterile filtered (0.2 µm)

E: FORMULATION OF PSC-PD ADSORBED CONJUGATE VACCINE

Washing of $AlPO_4$

In order to optimize the adsorption of PSC-PD conjugate on $AlPO_4$, the $AlPO_4$ is washed to reduce the PO_4^{3-} concentration:

- AlPO4 is washed with NaCl 150 mM and centrifuged (4x),
- the pellet is then resuspended in NaCl 150 mM,
- filtrated (100 µm) and
- the filtrate is heat sterilized

This washed $AlPO_4$ is referred to as WAP (washed autoclaved phosphate).

Formulation process

The PSC-PD conjugate bulk is adsorbed on AlPO4 WAP before the final formulation of the finished product. Formulation of PSC-PD adsorbed conjugate vaccine is shown in Figure 1.

Final composition/dose

- PSC-PD:	10 µg PS
- AlPO4 WAP:	0.25 mg Al 3+
- NaCl:	150 mM
- 2-phenoxy-ethanol:	2.5 mg
- Water for Injection:	to 0.5 ml
- pH:	6.1

F: PRECLINICAL INFORMATION

Immunogenicity of polysaccharide conjugate in mice

The immunogenicity of the PSC-PD conjugate has been assessed in 6- to 8-weeks-old Balb/C mice. The plain (unadsorbed) conjugate or the conjugate adsorbed onto AlPO4 was injected as a monovalent vaccine. Anti-PSC antibodies induced were measured by ELISA whilst functional antibodies were analysed using the bactericidal test, both methods being based on the CDC (Centers for Disease Control and Prevention, Atlanta, USA) protocols. Results from two different experiments performed to assess the response versus the dose and adjuvant ($AlPO_4$) effect are presented.

Dose-range experiment

In this experiment, the PSC-PD was injected twice (two weeks apart) in Balb/C mice. Four different doses of conjugate formulated on AlPO4 were used: 0.1 - 0.5 - 2.5 and 9.6 µg/animal. The mice (10/group) were bled on days 14 (14 Post I), 28 (14 Post II) and 42 (28 Post II). Geometric mean concentrations (GMCs) of polysaccharide C specific antibodies measured by ELISA were expressed in µg IgG/ml using purified IgG as reference Bactericidal antibodies were measured on pooled sera and titres expressed as the reciprocal of the dilution able to kill 50 % of bacteria, using the N. meningitidis C11 strain in presence of baby rabbit complement.

The dose-response obtained show a plateau from the dose of 2.5 µg. Results indicate that there is a good booster response between 14 Post I and 14 Post II. Antibody levels at 28 Post II are at least equivalent to those at 14 Post II. Bactericidal antibody titres are concordant with ELISA concentrations and confirm the immunogenicity of the PSC-PD conjugate.

Effect of adjuvant

In this experiment, one lot of PSC-PD conjugate formulated on AlPO4 was assessed, the plain (non-adjuvanted) conjugate was injected for comparison. 10 mice/group were injected twice, two weeks apart, by the subcutaneous route, with 2

μg of conjugate. Mice were bled on days 14 (14 Post I), 28 (14 Post II) and 42 (28 Post II), and ELISA (Figure 28) and functional antibody titres measured (only on 14 Post II and 28 Post II for the bactericidal test). The AlPO4 formulation induces up to 10 times higher antibody titres as compared to the non-adjuvanted formulations.

Conclusions

The following general conclusions can be made from the results of the experiments described above:

- PSC-PD conjugate induces an anamnestic response demonstrating that PSC, when conjugated, becomes a T cell dependent antigen.
- Anti-PSC antibody concentrations measured by ELISA correlate well with bactericidal antibody titres showing that antibodies induced by the PSC-PD conjugate are functional against N. meningitidis serogroup C.
- Approximately 2.5 μg of conjugate adsorbed onto AlPO4 appears to elicit an optimum antibody response in mice.
- The CDAP chemistry appears to be a suitable method for making immunogenic PSC-PD conjugates.

EXAMPLE 2

PREPARATION OF A POLYSACCHARIDE FROM NEISSERIA MENINGITIDIS SEROGROUP A – PD CONJUGATE

A dry powder of polysaccharide A (PS A) is dissolved during one hour in NaCl 0.2 M solution to a final concentration of 8 mg/ml. pH is then fixed to a value of 6 with either HCl or NaOH and solution thermoregulated at 25°C. 0.75 mg CDAP/mg PSA (a preparation to 100 mg/ml acetonitrile) is added to the PSA solution. After 1.5 minute without pH regulation, NaOH 0.2 M is added to obtain a pH of 10. 2.5 minutes later, protein D (concentrated to 5 mg/ml) is added according to a PD/PS A ratio of approximately 1. A pH of 10 is maintained during the coupling reaction period of 1 hour. Then, 10 mg glycine (2 M pH 9.0)/mg PS A is added and pH regulated at a value of 9.0 during 30 minutes at 25°C. The mixture is then conserved overnight at 4°C before purification by an exclusion column

chromatography (Sephacryl S400HR from Pharmacia). The conjugate elutes first followed by unreacted PD and by-product (DMAP, glycine, salts). The conjugate is collected and sterilized by a 0.2 μm filtration on a Sartopore membrane from Sartorius.

EXAMPLE 3

IN VITRO CHARACTERISATIONS

The major characteristics are summarized in the table here below.

N°	Conjugate description	Protein and PS content (μg/ml)	PS/protein ratio (w/w)	Free Protein (%)	Free PS (%)
1	PS C - PD NaOH for pH regulation	PD : 210 PS : 308	1/0.68	< 2	8-9
2	PS C - PD TEA for pH regulation	PD : 230 PS : 351	1/0.65	< 2	5-6
3	PS A - PD NaOH for pH regulation	PD : 159 PS : 149	1/1.07	5	5-9

In vivo results

Balb/C mice were used as animal model to test the immunogenicity of the conjugates. The conjugates were adsorbed either onto $AlPO_4$ or $Al(OH)_3$ (10 μg of PS onto 500 μg of Al^{3+}) or not adsorbed. The mice were injected as followed : 2 injections at two week intervals (2 μg PS/injection).

From these results, we can conclude first that free PS influence greatly the immune response. Better results have been obtained with conjugates having less than 10 % free PS.

The formulation is also important. $AlPO_4$ appears to be the most appropriate adjuvant in this model.

The conjugates induce a boost effect which is not observed when polysaccharides are injected alone.

Conclusions

Conjugates of N. Meningitidis A and C were obtained with a final PS/protein ratio of 1 and 0.6-0.7 (w/w) respectively.

Free PS and free carrier protein were below 10 % and 15 % respectively.

Polysaccharide recovery is higher than 70 %.

EXAMPLE 4 VACCINE COMPONENTS:

S.pneumoniae capsular polysaccharide:

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1,3,4,5,6B, 7F, 9V,14,18C,19F and 23F which were made essentially as described in EP72513.

Each polysaccharide is activated and derivatized using the CDAP chemistry and conjugated to the protein carrier.

All the polysaccharides are conjugated in their native form,except for the serotype 3.Its size was reduced by micro-fluidization.

Protein carrier:

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in E.coli.

Chemistry:

Activation and coupling chemistry:

The activation and coupling conditions are specific for each polysaccharide .These are given in Table 1.

Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile ,CDAP (CDAP/PS ratio:0.75 mg/mg PS) was added to the polysaccharide solution.1.5 minute later,0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2minutes at 25°C.Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour.

Then, the reaction was quenched with glycine for 30 minutes at 25°C and overnight at 4°C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein contents of the eluted fractions were determined .The conjugates were pooled and sterile filtered on a 0.22μm sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

Characterization:

Each conjugate was characterized and meet the specifications described in Table 2.

Polysaccharide and protein content (μg/ml):

The polysaccharide content was measured by the Resorcinol test and the protein content by the Lowry test. The final PS/PD ratio(w/w) is determined by the ratio of the concentrations.

Residual DMAP content (ng/μg PS):

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed and validated at SB.

Free polysaccharide content (%):

The free polysaccharide content on conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

Antigenicity:

The antigenicity on the same conjugates was analyzed in a sandwich-type ELISA wherein the capture and the detection of antibodies were α-PS and α-PD respectively.

Free protein content (%):

The level of "free" residual protein D was determined by using a method with SDS treatment of the sample. The conjugate was heated 10 min at 100°C in presence of SDS 0.1 % and injected on a SEC-HPLC gel filtration column (TSK 3000-PWXL).As protein D is dimmer, there is a risk to overestimate the level of "free" protein D by dissociation the structure with SDS.

Molecular size (K_{av}):

The molecular size was performed on a SEC-HPLC gel filtration column (TSK 5000-PWXL).

Stability:

The stability was performed on a HPLC-SEC gel filtration (TSK 6000-PWXL) on conjugates kept at 4°C and stored for 7 days at 37°C.

The 11-valent characterization is given in Table 2

The protein conjugates can be adsorbed onto aluminium phosphate and pooled to form the final vaccine.

Figure 1 : Formulation of PSC-PD adsorbed conjugate vaccine

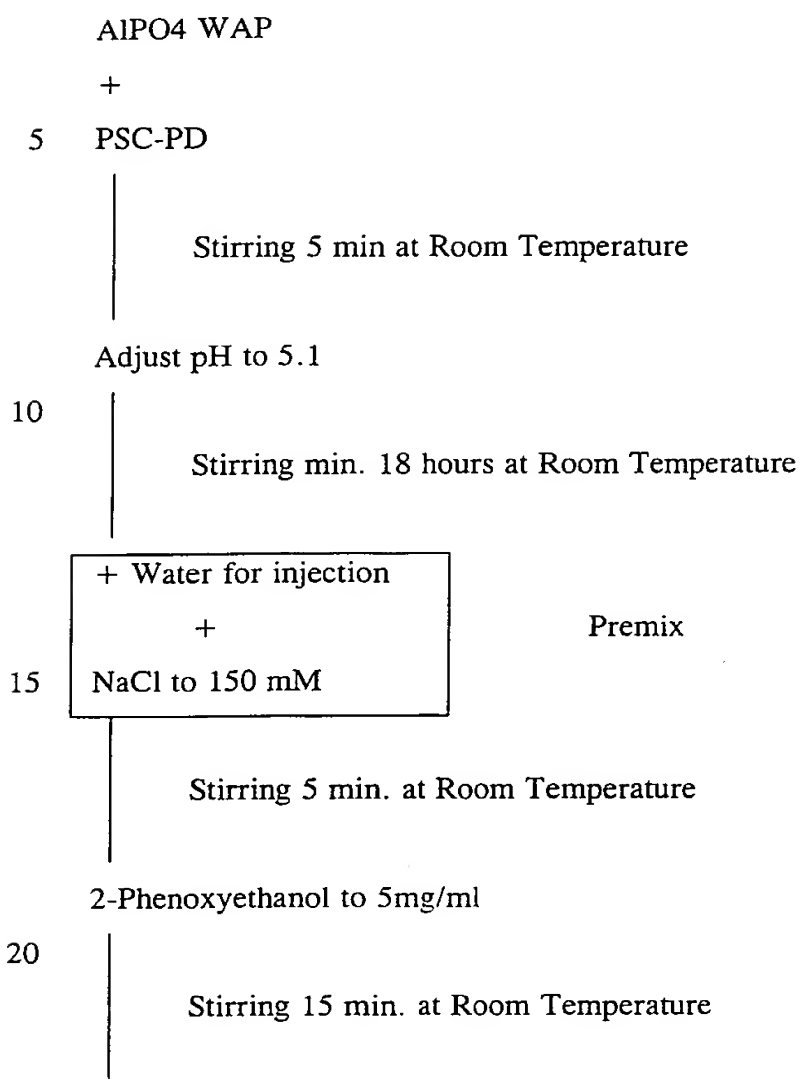

Table 1

Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

Serotype	1	3 (μfluid.)	4	5	6B	7F
PS conc.(mg/ml)	2.0	3.0	2.0	7.5	5.4	3.0
PS dissolution	NaCl 2M	NaCl 2M	H_2O	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/1	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0

Serotype	9V	14	18C	19F	23F
PS conc.(mg/ml)	2.5	2.5	2.0	4.0	3.3
PS dissolution	NaCl 2M	NaCl 2M	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1	1/0.5	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	10/9.5/9.0	9.0/9.0/9.0

TABLE 2

Criteria	D01PDJ227	D03PDJ236	D4PDJ228	D5PDJ235	D6PDJ209
Ratio PS/Prot (w/w)	1/0.66	1/1.09	1/0.86	1/0.86	1/0.69
Free polysac. content (%) <10 %	1	1	7	9	0
Free protein content (%) <15 %	8	<1	19	21	9
DMAP content (ng/μg PS) < 0.5 ng/μg PS	0.2	0.6	0.4	1.2	0.3
Molecular size (K_{av})	0.18	0.13	0.12	0.11	0.13
Stability	no shift	no shift	no shift	low shift	no shift

	D07PDJ225	D09PDJ222	D14PDJ202	D18PDJ221	D19PDJ206	D23PDJ212
Ratio PS/Prot (w/w)	1/0.58	1/0.80	1/0.68	1/0.62	1/0.45	1/0.74
Free polysac. content (%) <10 %	1	<1	<1	4	4	0
Free protein content (%) <15 %	8	0.3	3	21	10	12
DMAP content (ng/μg PS) <0.5 ng/μg PS	0.1	0.6	0.3	0.2	0.1	0.9
Molecular size (K_{av})	0.14	0.14	0.17	0.10	0.12	0.12
Stability	no shift	no shift	no shift	no shift	shift	no shift

Claims

1. A polysaccharide conjugate antigen comprising a polysaccharide antigen derived from a pathogenic bacterium conjugated to protein D from *Haemophilus influenzae* or a fragment thereof.

2. A polysaccharide conjugate as claimed in claim 1 wherein the polysaccharide antigens are selected from the Vi polysaccharide from *Salmonella typhi*, meningococcal polysaccharides including type A, C, W135 and Y), the polysaccharide and modified polysaccharides of group B meningococcus, polysaccharides from *Staphylococcus aureus*, polysaccharides from *Streptococcus agalactae* and *Streptococcus pneumoniae,* polysaccharides from Mycobacteria, [eg *Mycobacterium tuberculosis,* such as mannophosphoinisitides trehaloses, mycolic acid, mannose capped arabinomannans], the capsule therefrom and arabinogalactans, polysaccharide from *Cryptococcus neoformans*, the lipopolysaccharides of non-typeable *Haemophilus influenzae*, the capsular polysaccaharide from *Haemophilus influenzae b*, the lipopolysaccharides of *Moraxella catharralis*, the lipopolysaccharides of *Shigella sonnei*, the lipopeptidophosphoglycan (LPPG) of *Trypanosoma cruzi*, the cancer associated gangliosides GD3, GD2, the tumor associated mucins, especially the T-F antigen, and the sialyl T-F antigen, and the HIV associated polysaccharide that is structurally related to the T-F antigen.

3. An immunogenic composition comprising a plurality of polysaccharide conjugate antigens as claimed in claim 1 or 2.

4. An immunogenic composition as claimed in claim 3 comprising *Streptococcus pneumoniae* polysaccharide antigens from at least four *Streptococcus pneumoniae* serotypes.

5. An immunogenic composition as claimed in claim 4, wherein the polysaccharide antigens are derived from serotypes 6B, 14, 19F and 23F.

6. An immunogenic composition as claimed in any one of claims 3 to 5 comprising at least 11 polysaccharide antigen-conjugates derived from different streptococcus peneumoniae serotypes.

7. An immunogenic composition as claimed in any of claims 3 to 6 additionally comprising a *Streptococcus pneumoniae* protein.

8. An immunogenic composition comprising a protein D – Neisseria Meningitidis polysaccharide antigen conjugate.

9. An immunogenic composition as claimed in claim 8 wherein the polysaccharide antigen is derived from serotype A or C or a combination thereof.

10. An immunogenic composition comprising a protein D – *Haemophilius influenzae b* polysaccharide antigen conjugate.

11. An immunogenic composition as claimed in any of claims 8 to 10 wherein the polysaccharide antigens include antigens derived from Neisseria Meningitidis and *Haemophilius influenzae b* antigen.

12. An immunogenic composition as claimed herein additionally comprising an adjuvant.

13. An immunogenic composition as claimed in claim 12 wherein the polysaccharide – protein D conjugate is adsorbed onto aluminium phosphate.

14. An immunogenic composition as claimed in claim 13 wherein the adjuvant is a preferential inducer of a TH1 type of response.

15. An immunogenic composition as claimed herein for use in medicine.

16. A method of producing a polysaccharide - protein D conjugate, comprising activating said polysaccharide and coupling it to protein D.

17. A method of treating a patient suffering from or susceptible to infection from a pathogen bacterium comprising administering an effective amount of an immunogenic composition as claimed herein.

4 EP00/02467

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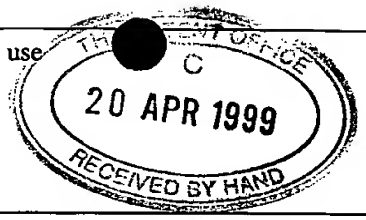

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Corporate Name SmithKline Beecham Biologicals s.a.

Country *(and State of incorporation, if appropriate)* Belgium

2b If you are applying as an individual or one of a partnership please give in full:

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Forenames

2c **In all cases,** please given the following details:

Address: Rue de l'Institut 89, B-1330 Rixensart, Belgium

UK postcode *(if applicable)*

Country Belgium
ADP number ~~**5808974002**~~ *(if known)*

05781117001.

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Novel Compositions

This invention relates to novel vaccine formulations, methods for preparing them and their use in therapy. In particular the present invention relates to combination vaccines for administration to children and the elderly.

Streptococcus pneumoniae is a gram positive bacteria responsible for considerable morbidity and mortality, particularly in the young and aged. Expansive colonisation of the respiratory tract, and middle ear, especially in young children, is the single most common cause for hospital visits in the US. The bacteria may become invasive, infecting the lower lungs and causing pneumonia. The rate of pneumococcal pneumonia in the US for persons over 60 years of age is estimated to be 3 to 8 per 100,000. In 20% of cases this leads to bacteremia, and other manifestations such as meningitis, with a mortality rate close to 30% even with antibiotic treatment. There are 90 known serotypes of *Streptococcus pneumoniae* which are determined by the structures of the capsular polysaccharide surrounding the bacteria, and this is its major virulence factor.

A 17 - valent pneumococcal vaccine (Moniarix) is known, based on the purified polysaccharides of the pneumococcal serotypes most commonly involved in invasive disease. The method of purification of these polysaccharides was disclosed in European Patent 72513 B1. Vaccine efficacy trials with lower valent vaccines demonstrated a 70 to 90% efficacy with respect to serotypes present in the combination. Case controlled studies in the US in persons >55 years using a 14 valent vaccine demonstrated 70% efficacy (Mills, O.F., and Rhoads, G.G; J. Clin. Epidemiol. (1996); Vol.49(6) 631-636). Inclusion of additional polysaccharides (to make a 23-valent pneumococcal vaccine) were accepted on the basis of an adequate serological response, even though there was clinical efficacy data lacking (K. R. Brown In 'Combined.Vaccines and Simultaneous Administration', Ed. Williams et al. New York Academy of Sciences 1995 pp 241-249).

Simultaneous immunisation with a 23-valent pneumococcal vaccine (Pnu-Immune 23 - Lederle USA) and influenza vaccine (Fluarix) has been studied (TJ Fletcher, TW Tunnicliffe, K Hammond, K Roberts, JG Ayres; (1997) Br. Med. J. 314: 1663) and no significant immunological differences were noted between simultaneous immunisation, or immunisations one month apart.

Pneumococcal polysaccharides can be rendered more immunogenic in infants by chemically coupling them to protein carriers, and clinical efficacy trials are being performed to verify this concept for efficacy in preventing infant Otitis media.

Infants primed with a 7 to 11-valent conjugate pneumococcal vaccine may be boosted with the 23-valent plain polysaccharide pneumococcal vaccine in order to increase the serotype coverage and IgG concentrations (Block, SL, JA. Hedrick, R.A. Smith, R.D. Tyler, M. Giordani, M.D. Blum, J. Sadoff, E. Keegan; Abstract G-88, 37th ICAAC, Toronto (1997); Anderson, E.L., Kennedy, D.J., Geldmacher, K.M., Donnelly, J., Mendelman, P; The Journal of Paediatrics, May 1996. Vo. 128, n°5, Part 1, 649-653).

Respiratory Syncytial virus (RSV) occurs in seasonal outbreaks, peaking during the winter in temperate climates and during the rainy season in warmer climates (DeSilva LM & Hanlon MG. Respiratory Syncytial Virus: a report of a 5-year study at a children's hospital. *J Med Virol* 1986;**19**:299-305). Wherever the area, RSV tends to have a regular and predictable pattern and other respiratory viral pathogens that occur in outbreaks are rarely present concurrently (Glezen WP & Denny FW. Epidemiology of acute lower respiratory disease in children. *N. Engl. J. Med.* 1973;**288**:498-505).

RSV infection is almost certainly underdiagnosed in adults, in part because it is considered to be an infection of children. Consequently, evidence of the virus in adults is not sought in order to explain respiratory illness. In addition, RSV is difficult to identify in nasal secretions from individuals who have some degree of partial immunity to the virus, as do the large majority of adults. Young to middle-age adults typically develop a persistent cold-like syndrome when infected with RSV. Elderly individuals may develop a prolonged

respiratory syndrome which is virtually indistinguishable from influenza, with upper respiratory symptoms which may be accompanied by lower respiratory tract involvement, including pneumonia. Institutionalised elderly populations are of particular concern, because they comprise large numbers of susceptible individuals clustered together. The spread of infection through such a population, many of whom have multiple medical problems which may predispose them to a more severe course of the disease, is difficult to control.

Furthermore, reports of recent studies evaluating the impact of RSV infection as a cause of hospitalisation in adults and in community dwelling healthy elderly further point to an important role of RSV infection in severe lower respiratory tract disease in these populations (Dowell SF, Anderson LJ, & Gary Jr HE, *et al. J. Infect Dis* 1996;**174**:456-462; Falsey AR, Cunningham CK, & Barker WH, *et al. J. Infect Dis* 1995;**172**:389-394). Dowell identified RSV as one of the four most common pathogens causing severe lower respiratory tract disease resulting in hospitalisation of adults (Dowell SF, Anderson LJ, & Gary Jr HE, *et al. J. Infect Dis* 1996;**174**:456-462). Falsey demonstrated that serious RSV infections in elderly persons are not limited to nursing homes or outbreak situations. Rather, RSV infection is a predictable cause of serious illness among elderly patients residing in the community. Similar to hospitalisations for influenza A, those related to RSV infections were associated with substantial morbidity, as evidenced by prolonged hospital stays, high intensive care admission rates, and high ventilatory support rates (Falsey AR, Cunningham CK, & Barker WH, *et al. J. Infect Dis* 1995;**172**:389-394).

These studies point to the medical and economic need for an effective vaccine which can prevent severe complications of RSV infection in infants, adults and both community dwelling healthy and institutionalised elderly.

SmithKline Beecham has undertaken the development of an RSV vaccine based on the extracellular domain of the F and G surface glycoproteins of RSV strain A. The use of an adjuvant is of primary importance for a subunit recombinant vaccine. Alum is the only adjuvant currently licensed for human use. However, Alum has a limited adjuvant effect

on the humoral response, and is known to induce mainly a TH2 cellular response (Byars NE, Nakano G, & Welch M, *et al. Vaccine* 1991;9:309-318). It has been found, however, that 3 De-O-acylated monophosphoryl lipid A (3D-MPL) in combination with Alum improves the humoral response and stimulates preferentially a TH1-type immunity. The Aluminium salt with 3D-MPL adjuvant is denoted Alum/3D-MPL.

The present invention provides a vaccine composition comprising:

(a) one or more *Streptococcus pneumoniae* polysaccharides either conjugated to a protein or peptide, or non-conjugated; and
(b) an RSV antigen
in combination with an adjuvant which is a preferential stimulator of TH1 cell response.

The trend towards combination vaccines has the advantage of reducing discomfort to the recipient, facilitating scheduling, and ensuring completion of regiment; but there is also the concomitant risk of reducing the vaccine's efficacy. It would be, therefore, advantageous to make vaccine combinations which meet the needs of a population, and which, in addition, do not interfere with each other. It would be of further advantage if the combination of the vaccines results in synergy with resulting improvement of one or both vaccines efficacy, or improved correlates of protections for one or both vaccines. This is achieved by the vaccine composition of the invention which is of great benefit for administration to children or the elderly who may be particularly at risk of *Streptococcus pneumoniae*, and/or RSV infection.

Optionally the vaccine composition of the invention additionally comprises one or more of a number of other antigens such as an antigen against *influenza* virus. Currently available influenza vaccines include whole inactivated virus vaccines, split particle vaccines, and subunit vaccines. Inactivated influenza vaccines, of all kinds, are usually trivalent vaccines. They contain antigens derived from two influenza A virus strains and one

influenza B strain. A standard 0.5ml dose of most of them contain 15μg of haemagglutinin component from each strain.

The procedure for determining the strains to be incorporated into an influenza vaccine is a complex process which involves collaboration between the World Health Organisation, national health authorities and vaccine manufacturers.

There are two conjugation methods generally used for producing immunogenic polysaccharide constructs: (1) direct conjugation of carbohydrate and protein; and (2) indirect conjugation of carbohydrates and protein via a bifunctional linker or spacer reagent. Generally, both direct and indirect conjugation require chemical activation of the carbohydrate moiety prior to derivatisation. See for example US 5,651,971 and Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds), Vol. 10, 48 - 114 (1989).

The *Streptococcus pneumoniae* polysaccharides, if conjugated, are conjugated to either a protein or a peptide. Polysaccharide antigens have been conjugated to a number of T helper proteins. These provide T-helper epitopes. Representative proteins include Diphtheria Toxoid, Tetanus toxoid, and protein D or its lipidated derivative lipoprotein D from Heamophilius influenzae B. Other suitable protein carriers include, Diphtheria Crm 197 and the major non structural protein from influenzae, NS1 (particularly amino acid 1-81).

The *Streptococcus pneumoniae* polysaccharide in the composition of the invention is preferably a vaccine with a number of valencies, preferably at least an 11-valent vaccine, for example a 17- or 23-valent vaccine. Most preferably the 23-valent pneumococcal polysaccharide or the 11-valent conjugate vaccine.

Suitable RSV antigens for inclusion in vaccines include an inactivated RSV virus, such as a formalin inactivated RSV virus, or antigens derived from the RSV virus, preferably human RSV envelope glycoproteins, such as the RSV F or G protein or immunogenic fragments

thereof as disclosed for example in US Patent 5149650, or a chimeric polypeptide comprising at least one immunogenic fragment from both RSV F and G proteins, advantageously an RSV FG chimeric protein as disclosed for example in US patent 5194 595 preferably expressed from CHO cells.

The RSV antigen in the composition of the invention is preferably an RSV FG chimeric protein.

Adjuvants which are capable of preferential stimulation of the TH1 cell response are described in International Patent Application No. WO 94/00153 and WO 95/17209.

3 De-O-acylated monophosphoryl lipid A (3D-MPL) is one such adjuvant. This is known from GB 2220211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in International Patent Application No. 92/116556.

Preferably, the size of the particles of 3D-MPL are small enough to be sterile filtered through a 0.22micron membrane (as described in European Patent number 0689454).

3D-MPL will be present in the range of 10μg - 100μg preferably 25-50μg per dose wherein the antigen will typically be present in a range 2-50μg per dose.

Another preferred adjuvant comprises QS21, an Hplc purified non-toxic fraction derived from the bark of Quillaja Saponaria Molina. Optionally this may be admixed with 3 De-O-acylated monophosphoryl lipid A (3D-MPL), optionally together with an carrier.

QS21 is a Hplc purified non-toxic fraction of a saponin from the bark of the South American tree Quillaja Saponaria Molina and its method of its production is disclosed (as QS21) in US patent No. 5,057,540.

Non-reactogenic adjuvant formulations containing QS21 have been described previously (WO 96/33739). Such formulations comprising QS21 and cholesterol have been shown to be successful TH1 stimulating adjuvants when formulated together with an antigen. Thus vaccine compositions which form part of the present invention may include a combination of QS21 and cholesterol.

Further adjuvants which are preferential stimulators of TH1 cell response include immunomodulatory oligonucleotides, for example unmethylated CpG sequences as disclosed in WO 96/02555.

Combinations of different TH1 stimulating adjuvants, such as those mentioned hereinabove, are also contemplated as providing an adjuvant which is a preferential stimulator of TH1 cell response. For example, QS21 can be formulated together with 3D-MPL. The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1:5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5 : 1 to 1 : 1 3D MPL: QS21.

Preferably a carrier is also present in the vaccine composition according to the invention. The carrier may be an oil in water emulsion, or alum.

A preferred oil-in-water emulsion comprises a metabolisible oil, such as squalene, alpha tocopherol and tween 80. Additionally the oil in water emulsion may contain span 85 and/or lecithin and/or tricaprylin.

In a preferred aspect aluminium hydroxide (alum) or aluminium phosphate will be added to the composition of the invention to enhance immunogenicity.

In a particularly preferred aspect the antigens in the vaccine composition according to the invention are combined with 3D-MPL and alum.

Typically for human administration QS21 and 3D-MPL will be present in a vaccine in the range of 1 μg - 200 μ g, such as 10-100 μ g, preferably 10 μ g - 50 μ g per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal or less than 1 as this provides amore stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Non-toxic oil in water emulsions preferably contain a non-toxic oil, e.g. squalane or squalene, an emulsifier, e.g. Tween 80, in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210.

The vaccine of the present invention will contain an immunoprotective quantity of the antigens and may be prepared by conventional techniques.

Vaccine preparation is generally described in Pharmaceutical Biotechnology, Vol.61 Vaccine Design - the subunit and adjuvant approach, edited by Powell and Newman, Plenum Press, 1995; New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Encapsulation within liposomes is described, for example, by Fullerton, U.S. Patent 4,235,877. Conjugation of proteins to macromolecules is disclosed, for example, by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 mg of protein, preferably 2-100 mg, most preferably 4-40 mg. An optimal amount for a particular vaccine can be

ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

In addition to vaccination of persons susceptible to *Streptococcus pneumoniae* or RSV infections, the pharmaceutical compositions of the present invention may be used to treat, immunotherapeutically, patients suffering from the said infections.

In a further aspect of the present invention there is provided a method of manufacture as herein described, wherein the method comprises mixing a *Streptococcous pneumoniae* antigen and an RSV antigen with a Th-1 inducing adjuvant, for example 3D-MPL and, preferably, a carrier, for example alum.

If desired, other antigens may be added, in any convenient order, to provide multivalent vaccine compositions as described herein.

In the examples that follow, evaluation of the effect of combining the RSV vaccine candidate, FG with the commercially available 23-valent pneumococcal vaccine (Pneumune from Wyeth-Lederle) in a mouse model was carried out. This model tested a combined immunisation where the vaccines are physically mixed and injected in the same site. The results of this study demonstrate a mutual synergy, resulting in improved responses for certain immunological markers for both vaccines.

EXAMPLES

1. Formulation process:

H_2O-diluted FG antigen (2μg:1/10 HD) was adsorbed for 15 min on 50 μg of $Al(OH)_3$. When used, 5μg of 3D-MPL were added to the preparation as a suspension of 100 nm particles and incubated for 30 min. Formulations were then buffered with 10-fold concentrated PBS pH 7.4. When used, the 23 valent pneumococcal vaccine (11.5μg:1/50 HD) was added 15 min. after the addition of the buffer solution. For groups without FG the same formulation sequence was followed so that the 3D-MPL was first adsorbed on $Al(OH)_3$ before adding the 23 valent pneumococcal vaccine. Fifteen minutes after the addition of the concentrated buffer or the 23 valent pneumococcal vaccine phenoxyethanol (5mg/ml) was added to the formulations as preservative.

All incubations were carried out at room temperature with agitation. The formulations were prepared simultaneously for the 2 injections with a 7-day maturation of the finalised formulations before the first injection.

Composition of formulation constituents:

COMPONENT	BATCH NUMBER	CONCENTRATION (μg/ml)	BUFFER
FG	54/023	265	10 PO4, 150NaCl pH6.8
Al $(OH)_3$	96A0089	10380	H_2O
23-Valent	Pneumune	1150	saline
3D-MPL	109	964	H_2O

2. Immunization protocol:

11 groups of 10 mice were immunised by different routes (50 μl) at days 0 and 28 with various formulations (see ***Table 1***). Group 7 and 8 were immunised with live RSV by the intra-nasal route (60μl). Sera were obtained at days 28 (28 d Post I) and 42 (14 d Post II). On day 42, spleen and node cells were taken from 5 mice of groups 4, 5, 6, 7, 8, 9, as well as from 5 naïve Balb/c mice (group 1, not immunised).

3. Humoral response.

3.1. Anti-FG antibodies:

All humoral results were performed for 10 mice/group (individual response for the anti-FG titers and pooled sera for the isotype profile) and cellular results were presented for 5 mice/group.

Individual sera were obtained 28 days after the first Immunization and 14 days after the second immunisation and were tested for the presence of FG specific Ig antibodies and their isotype (IgG2a, IgG1) distribution.
The assay protocol was as follows: coating overnight at 4°C with 50 µl of purified FG 54/023 (1µg/ml) per well, saturation 1h at 37°C, incubation with sera 1h30 at 37°C, incubation with anti-mouse Ig biotin 1/1500 (or IgG1, IgG2a biotin 1/1000) 1h30 at 37°C, incubation with strepta-peroxydase 1/2500 30 min at 37°C, incubation with OPDA Sigma 15 min at RT, stop with H_2SO_4 2N.
OD were monitored at 490 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD. 95% confidence limits (IC 95%) were calculated for each group.

Individual sera obtained 14d Post II were tested for the presence of neutralising antibodies using the following protocol: 50 µl of serial two-fold dilutions of sera (first dilution 1/250) were incubated for 1 hour at 37°C with 50 µl of a mixture containing 500 pfu of RSV-A/Long (Lot 14) and guinea pig complement in a 96 well plate in duplicate. 100 µl of a HEp-2 cell suspension at 10^5 cells/ml were then added to each well and the plates were incubated for 4 days at 37°C in the presence of 5% CO_2.
The supernatants were then aspirated, and after addition of a 100 µl of a WST-1 preparation (dilution 1/12.5) the plates were further incubated for 24H at 37°C in the presence of 5% CO_2. The OD were monitored at 595 nm and the titers determined by linear regression (y=a.logx + b): titer = serum dilution giving 50% reduction of the maximal OD observed for the uninfected cells.
Controls in test included a pool of randomly chosen human sera (Human pool) and Sandoglobuline (lot 069, generic human IgG produced by Sandoz).

3.2. Anti-Pneumococcal Polysaccharide IgG:

Murine IgG to pneumococcal polysaccharides types 6B, 14, 19F and 23F was measured by ELISA in a method adapted from the CDC protocol. This protocol includes the addition of soluble cell wall polysaccharide (CPS) to the sera to inhibit the measurement of CPS antibodies. CPS is a phosphoryl choline containing teichoic acid common to all pneumococci. It is present under the capsule, and antibodies to it are only weakly

protective. Since CPS is linked to the capsular polysaccharide, there is usually 0.5 to 1% CPS contaminating the purified capsular polysaccharide used to coat the ELISA plates. Thus, measurement of the CPS antibodies can confound the interpretation ELISA results with respect to the capsular polysaccharide.
The ELISA was performed with polysaccharides coated at 20, 5, 10 and 20 μg/ml in carbonate buffer for types 6B, 14, 19F and 23F respectively. Sera was pre-mixed with the equivalent of 500 μg/ml CPS in undiluted sera, and incubated for 30 minutes before addition to the ELISA plate. Murine IgG was detected with Jackson ImmunoLab goat anti-murine IgG (H+L) peroxidase at 1:2000 dilution. The titration curves were referenced to polysaccharide specific murine monoclonal antibodies of known concentration for each serotype using logistic log comparison by SoftMax Pro. The monoclonals used were HASP4, PS14/4, PS19/5 and PS23/22 for types 6B, 14, 19F and 23F respectively. Due to the limited quantity of sera available, pooled sera was tested, thus statistical analysis is not available.

4. Cellular response

Spleen and lymph node cells were isolated 14d Post II from groups 4-9 and from naïve mice (Group 1) for use as a negative control for the FG-specific cellular response analysis. Samples were analysed for both FG-specific lymphoproliferation and cytokine (IFN-γ + IL-5) secretion.
Proliferation was evaluated after a 96h incubation of $4x10^5$ cells/well of 96 well plates with 200 μl of media containing 10 to 0.03 μg/ml of FG (3-fold dilutions). Upon ^{3}H-thymidine incorporation, the FG specific proliferation was measured following our standard protocol. Cytokine induction was evaluated after 96 h incubation of $2.5x10^6$ cells per well of 24 well with 1 ml of media containing 10μg to 0.01μg of FG (10-fold dilutions). Supernatants were then harvested to determine the quantity of IFN-γ and IL-5 induced by ELISA following our standard protocol.

Results

1. GROUPS.

10 groups received two immunisations of various formulations containing either the 23-valent vaccine or FG or a combination of both formulated with Aluminium hydroxide and 3D-MPL or Aluminium hydroxide. Group 1 constitutes the control for the CMI studies. Groups 2 and 9 will allow the evaluation of the immunogenicity of the 23 Valent pneumoccocal vaccine upon intraperitoneal immunization which was used in the literature for the evaluation of 23 Valent pneumoccocal vaccine, and upon IM immunization, Groups 3 and 10 allow a parallel analysis to groups 2 and 9 except that the 23 Valent pneumoccocal vaccine is formulated with Alum/3D-MPL. Groups 2 and 3 also constitute

controls for the impact of Alum/3D-MPL on the 23 Valent pneumoccocal vaccine when it is not combined to FG Alum/3D-MPL. Group 4 will allow the evaluation of the immune response induced upon IM Immunization of the combination of the 23 Valent pneumoccocal vaccine and FG Alum/3D-MPL. Groups 5 and 6 constitute a control for the evaluation of the impact of Alum/3D-MPL on FG when it is not combined with the 23 Valent pneumoccocal vaccine. The RSV live immunisations was a control for the immune response induced upon natural RSV IN infection (Groups 7). Finally, group 9 is a control for the impact of Alum/3D-MPL alone.

TABLE 1

Groups	Antigen	Adjuvant	Route
1	none	none	
2	23 Valent Pneumune(11.5μg)	none	IM
3	23 Valent Pneumune (11.5μg)	Alum/3D-MPL	IM
4	23 Valent Pneumune (11.5μg) / FG (2μg)	Alum/3D-MPL	IM
5	FG (2μg)	Alum/3D-MPL	IM
6	FG (2μg)	Alum	IM
7	RSV Live (Lot 14: 10^5PFU)	none	IN
8	none	Alum/3D-MPL	IM
9	23 Valent Pneumune (11.5μg)	none	IP
10	23 Valent Pneumune (11.5μg)	Alum/3D-MPL	IP

2. HUMORAL RESPONSE.

2.1. Anti-FG antibodies

- The analysis of specific anti-FG antibodies at 28 d Post I and 14 d Post II shows the induction of similar antibody responses upon Immunization with FG Alum/3D-MPL / 23-Valent (Group 4) or FG Alum/3D-MPL alone (Group 5) with slightly higher titers observed for FG Alum/3D-MPL / 23-Valent (***Figure 1***). The Immunization with FG Alum (Group 6) induces expectedly lower antibody titers at both time points. Statistical analysis shows that there is indeed a significant difference between the anti-FG Ig titers induced by Groups 4 -6 at 28 d Post I and 14 d Post II.

RESULTS

- The analysis of anti-RSVA neutralizing antibodies (***Figure 2***) presents a parallel profile as the one observed for the anti-FG Ig responses.

- Based on the above results, the anti-FG / anti RSV neutralizing antibody ratio (28d PostII) was calculated and showed that the ratio of FG Alum/3D-MPL / 23-Valent, FG Alum/3D-MPL and FG Alum compares to the ratio induced by the RSV IN group (***Figure 3***).

- An in depth statistical analysis on individual anti-FG Ig, IgG1 and IgG2a isotype titers showed that the addition of the 23-valent vaccine to FG Alum/3D-MPL maintains the Ig and IgG1 responses and increases significantly the IgG2a responses (***Figure 4A***). In addition, the analysis showed that the IgG1/IgG2a ratio and the IgG1 titer of FG Alum/3D-MPL / 23 valent and FG Alum/3D-MPL were similar although significantly different from FG Alum (***Figure 4B***). The three formulations had significant differences in their IgG2a titers.

2.2. Anti-Pneumococcal Polysaccharide IgG

While mice and other rodents may produce IgM against polysaccharide immunogens, they do not normally produce IgG against polysaccharides. This is because their immune system lacks the signals to induce isotype switching to a T-independent antigen such as a polysaccharide. Thus, the measurement of anti-polysaccharide IgG in mice is a sensitive test for the induction of an improved T-independent immune response.

The concentrations of IgG induced against four of the 23 polysaccharides are presented in ***Figure 5***. As we had noted in other experiments, no IgG was produced against polysaccharide types 6B and 23F. However, there was measurable IgG produced against types 14 and 19F.

- Groups that did not contain 23-Valent vaccine did show any detectable IgG to polysaccharides 14 and 19F, confirming the specificity of the measurements.

- A comparison of the route of immunisation reveals that when the 23-valent vaccine is adjuvanted with Alum/3D-MPL, the intra-muscular route appears to be better than inter-peritoneal for type 19F, whereas the reverse is true for type 14. With plain 23-Valent, there does not seem to be a significant difference.

- 23Valent + Alum/3D-MPL induces greater IgG for types 14 and 19F, in both IM and IP immunisation routes when compared to 23-Valent alone. When combined with FG,

however, there is a reduction in the IgG response. Nevertheless, the IgG response to 19F in 23-Valent+FG/Alum/3D-MPL is still greater than that induced by 23-Valent alone (compare groups 3 and 4), and the response to type 14 is improved.

3. CELLULAR RESPONSE.

- The induced FG-specific lymphoproliferation did not show any difference between FG Alum/3D-MPL +/- 23 Valent and FG Alum, both in spleen cells and in lymph node cells (***Figure 6***).

- The analysis of the production of IL-5 and IFN-γ suggests that the mixture of the 23 valent vaccine with FG Alum/3D-MPL does not hamper the production of IFN-γ but could increase somewhat the production of IL-5 (***Figure 7***). This observation is confirmed by a 2 to 8 fold difference in the IFN-γ / IL-5 ratio observed at two doses of FG (10 and 1 µg FG/ml) used for *in vitro* restimulation. However, the FG Alum/3D-MPL / 23-valent vaccine still induces a much higher IFN-γ / IL-5 ratio than FG Alum.

CONCLUSIONS

The analysis of the induced anti-FG Ig specific and anti RSVA neutralizing antibody titers shows that the combination of FG Alum/3D-MPL with the 23 valent pneumococcal vaccine does not hamper the induction of the response observed with FG Alum/3D-MPL alone. The quality of the response measured by the anti-FG/anti-RSVA neutralizing antibody ratio also remains unchanged and close to the ratio observed upon natural infection with RSV true the IN route.

Analysis of the induced FG specific cell mediated response suggest that the addition of the 23 valent vaccine to FG Alum/3D-MPL does not affect the induction of lymphoproliferation in both spleen cells and lymph node cells. Furthermore, the induction of a Th1 type response typically observed with FG Alum/3D-MPL is not hampered by the addition of the 23 valent as measured by in vitro cytokine production of FG specific spleen and lymphnode cells and appears to be enhanced when analysing the FG specific isotype antibody distribution. Indeed, the production of IFN-γ, marker of a Th1 response, remains unchanged upon addition of the 23 valent vaccine to FG Alum/3D-MPL while the production of Il-5, marker of a Th2 response is only slightly increased. Interestingly, the addition of the 23 valent vaccine to FG Alum/3D-MPL significantly increases the production of IgG2a antibodies, marker of a Th1 response, while it is not affecting the IgG1 (marker of a Th2 response) nor the IgG1/IgG2a response.

Thus the combination of FG Alum/3D-MPL and 23-valent vaccine does not affect the response observed with FG Alum/3D-MPL and therefore the advantages linked to the FG Alum/3D-MPL formulation versus FG Alum i.e., induction of high primary and secondary neutralizing antibody responses and a Th1 response as measured by the presence of IgG2a antibodies and the induction by high levels of IFN-γ are maintained.

Combination of 23Valent Pneumune with FG Alum/3D-MPL results in increased IgG production in 2 of 4 polysaccharides tested, but most dramatically for type 19F. 23-Valent alone with Alum/3D-MPL gave the highest IgG concentrations.

In conclusion, there is a favourable combination of 23-Valent pneumococcal vaccine with RSV FG Alum/3D-MPL in which there is a synergistic effect for both vaccines in that both vaccines show improvements in certain immunological tests, and no inhibition in any tests.

Claims

1. A vaccine composition comprising:

(a) one or more *Streptococcus pneumoniae* polysaccharides either conjugated to a protein or peptide, or non-conjugated; and

(b) an RSV antigen

in conjunction with an adjuvant which is a preferential stimulator of TH1 cell response.

2. A vaccine composition according to claim 1 in which the *Streptococcus pneumoniae* polysaccharide is conjugated preferentially to a protein from the group comprising; Protein D from Haemophilus influenzae B, a lipidated version thereof (lipoprotein D); Tetanus toxin, and Diphtheria Toxin.

3. A vaccine composition according to claim 1 or claim 2 which additionally comprises a carrier.

4. A vaccine composition according to any one of claims 1 to 3 in which the preferential stimulator of TH1-cell response is selected from the group of adjuvants comprising: 3D-MPL, 3D-MPL wherein the size of the particles of 3D-MPL is preferably about or less than 100nm, QS21, a mixture of QS21 and cholesterol, and a CpG oligonucleotide.

5. A vaccine composition according to claim 4 in which the preferential stimulator of TH1-cell response is 3D-MPL.

6. A vaccine composition according to any one of claims 1 to 5 in which the *Streptococcus pneumoniae* polysaccharide is a 23-valent pneumococcal polysaccharide vaccine.

7. A vaccine compostion according to any one of claims 1 to 5 in which the *Streptococcus pneumoniae* polysaccharide is an 11-valent conjugated pneumococcal polysaccharide vaccine.

8. A vaccine composition according to any one of claims 1 to 7 in which the RSV antigen is a human RSV envelope glycoprotein.

9. A vaccine composition according to any one of claims 1 to 8 in which the RSV antigen is chimeric FG or a fragment thereof.

10. A vaccine composition according to any one of claims 1 to 9 in which an influenza virus antigen is additionally present.

11. A vaccine composition according to any one of claims 1 to 10 in which the carrier is selected from the group comprising aluminium hydroxide, aluminium phosphate and tocopherol and an oil in water emulsion.

4

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Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: Vaccine

❷ Applicant's details

☐ **First or only applicant**

2a If you are applying as a corporate body please give:
Corporate Name: SmithKline Beecham Biologicals S.A.

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2b If you are applying as an individual or one of a partnership please give in full:

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2c **In all cases,** please given the following details:

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please give details below

Agent's name **KATHRYN L. PRIVETT**

Agent's address SmithKline Beecham
Corporate Intellectual Property
New Horizons Court
Great West Road
Brentford, Middlesex

Postcode TW8 9EP

Agent's ADP number S2009740V4

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❹ Reference number

4. Agent's or applicant's reference number *(if applicable)* KLP/MJL/ND/B45190

❺ Claiming an earlier application date

5. Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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Claim(s) 3 Description 28

Abstract [ ] Drawing(s)

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VACCINE

FIELD OF INVENTION

The present invention relates to the field of *Streptococcus pneumoniae* capsular polysaccharide antigen vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to.the field of pneumococcal capsular polysaccharide conjugate vaccines, and novel adjuvanted compositions thereof.

BACKGROUND OF INVENTION

Streptococcus pneumoniae is a Gram-positive bacteria that is pathogenic for humans, causing invasive diseases such as pneumonia, bacteremia and meningitis, and diseases associated with colonisation, such as acute Otitis media. It is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

Polysaccharide antigen based vaccines are well known in the art. Four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33 (accounting for at least 90% of pneumococcal blood isolates).

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet these vaccines have not been licensed for use in children less than two years of age because they are inadequately immunogenic in this age group [Peltola *et al.*(1984), N. Engl. J. Med. 310:1561-1566]. *Streptococcus pneumoniae* is the most common cause of invasive bacterial disease and otitis media in infants and young children. Likewise, aged adults mount poor responses to pneumococcal vaccines [Roghmann *et al.*, (1987), J. Gerontol. 42:265-270], hence the increased incidence of bacterial pneumonia in this population [Verghese and Berk, (1983) Medicine (Baltimore) 62:271-285].

Strategies, which have been designed to overcome this lack of immunogenicity in infants, include the linking of the polysaccharide to large immunogenic proteins, which provide bystander T-cell help and which induce immunological memory against the polysaccharide antigen to which it is conjugated. Examples of these proteins which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, CRM197 [a mutant of DT], and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD). Pneumococcal glycoprotein conjugate vaccines are currently being evaluated for safety, immunogenicity and efficacy in various age groups.

In addition, it is generally accepted that the protective efficacy of the commercialised pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component polysaccharide (Ed. Williams *et al.* New York Academy of Sciences 1995 pp. 241-249). Therefore further enhancement of antibody responses to the pneumococcal polysaccharides could increase the percentage of infants and elderly responding with protective levels of antibody to the first injection of polysaccharide or polysaccharide conjugate and could reduce the dosage and the number of injections required to induce protective immunity to infections caused by *Streptococcus pneumoniae*.

Since the early 20th century, researchers have experimented with a huge number of compounds which can be added to antigens to improve their immunogenicity in vaccine compositions [reviewed in M.F. Powell & M.J. Newman, Plenum Press, NY, "Vaccine Design – the Subunit and Adjuvant Approach" (1995) Chapter 7 "A Compendium of Vaccine Adjuvants and Excipients"]. Many are very efficient, but cause significant local and systemic adverse reactions that preclude their use in human vaccine compositions. Aluminium-based adjuvants (such as alum, aluminium hydroxide or aluminium phosphate), first described in 1926, remain the only immunologic adjuvants used in human vaccines licensed in the United States.

Aluminium-based adjuvants are examples of the carrier class of adjuvant which works through the "depot effect" it induces. Antigen is adsorbed onto its surface and when the composition is injected the adjuvant and antigen do not immediately dissipate in the blood stream – instead the composition persists in the local environment of the injection and a more pronounced immune response results. Such carrier adjuvants have the additional known advantage of being suitable for stabilising antigens that are prone to breakdown, for instance some polysaccharide antigens.

3D-MPL is an example of a non-carrier adjuvant. Its full name is 3-O-deacylated monophosphoryl lipid A (or 3 De-O-acylated monophosphoryl lipid A or 3-*O*-desacyl-4' monophosphoryl lipid A) and is referred to as 3D-MPL to indicate that position 3 of the reducing end glucosamine is de-O-acylated. For its preparation, see GB 2220211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. It was originally made in the early 1990's when the method to 3-O-deacylate the 4'-monophosphoryl derivative of lipid A (MPL) led to a molecule with further attenuated toxicity with no change in the immunostimulating activity.

3D-MPL has been used as an adjuvant either on its own or, preferentially, combined with a depot-type carrier adjuvant such as aluminium hydroxide, aluminium phosphate or oil-in-water emulsions. In such compositions antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of

antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1]. Precipitated aluminium-based adjuvants are preferred as they are the only adjuvants that are currently used in licensed human vaccines. Accordingly, vaccines containing 3D-MPL in combination with aluminium-based adjuvants are favoured in the art due to their ease of development and speed of introduction onto the market.

MPL (**non** 3-deacylated) has been evaluated as an adjuvant with several monovalent polysaccharide-conjugate vaccine antigens. Coinjection of MPL in saline enhanced the serum antibody response for four monovalent polysaccharide conjugates: pneumococcal PS 6B-tetanus toxoid, pneumococcal PS 12-diphtheria toxoid, and *S. aureus* type 5 and *S. aureus* type 8 conjugated to *Pseudomonas aeruginosa* exotoxin A [Schneerson *et al.* J. Immunology (1991) 147:2136-2140]. The enhanced responses were taught as being antigen-specific. MPL in an oil-in-water emulsion (a carrier type adjuvant) consistently enhanced the effect of MPL in saline due to the presence of MPL and antigen in the same particulate structure, and was considered to be the adjuvant system of choice for optimal delivery of other polysaccharide conjugate vaccines.

Devi *et al.* [Infect. Immun. (1991) 59:3700-7] evaluated the adjuvant effect of MPL (non 3-deacylated) in saline on the murine antibody response to a TT conjugate of *Cryptococcus neoformans* capsular polysaccharide. When MPL was used concurrently with the conjugate there was only a marginal increase in both the IgM- and IgG-specific response to the PS; however MPL had a much larger effect when administered 2 days after the conjugate. The practicality of using an immunization scheme that requires a delay in the administration of MPL relative to antigen, especially in infants, is questionable. The adjuvant effect of MPL with polysaccharides and polysaccharide-protein conjugates appears to be composition-dependent. Again, the incorporation of MPL in a suitable slow-release delivery systems (for instance using a carrier adjuvant) provides a more durable adjuvant effect and circumvents the problem of timing and delayed administration.

In summary, the state of the art has taught that, for particular polysaccharide or polysaccharide-conjugate antigens, where MPL or 3D-MPL is used as an adjuvant, it is advantageously used in conjuction with a carrier adjuvant (for instance the aluminium-based adjuvants) in order to maximise its immunostimulatory effect.

Surprisingly, the present inventors have found that for certain pneumococcal polysaccharide conjugates, the immunogenicity of the vaccine composition is significantly greater when the antigen is formulated with 3D-MPL alone rather than with 3D-MPL in conjunction with an aluminium-based carrier adjuvant. Furthermore the observed improvement is independent of the concentration of 3D-MPL used, and whether the particular conjugates are in a monovalent composition or whether they are combined to form a polyvalent composition.

SUMMARY OF THE INVENTION

Accordingly, the present invention provides an antigenic composition comprising one or more pneumococcal polysaccharide conjugates adjuvanted with 3D-MPL and substantially devoid of aluminium-based adjuvants, wherein at least one of the pneumococcal polysaccharide conjugates is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant.

Preferred embodiments provided are antigenic compositions comprising conjugates of one or more of the following pneumococcal capsular polysaccharides: serotype 4, 6B, 18C, 19F, and 23F. In such compositions, each of the polysaccharides are surprisingly more immunogenic in compositions comprising 3D-MPL alone compared with compositions comprising 3D-MPL and an aluminium-based adjuvant.

Thus is one embodiment of the invention there is provided a antigenic composition comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 4, 6B, 18C, 19F or 23F conjugated to an immunogenic protein and 3D-MPL adjuvant, wherein the composition is substantially devoid of aluminium-based adjuvants.

In a second embodiment, the present invention provides a combination antigenic composition substantially devoid of aluminium-based adjuvants and comprising 3D-MPL adjuvant and two or more pneumococcal polysaccharide conjugates chosen from the group consisting of: serotype 4; serotype 6B; serotype 18C; serotype 19F; and serotype 23F.

DESCRIPTION OF THE INVENTION

For the purposes of this invention, the term "pneumococcal polysaccharide conjugates of the invention" describes those conjugates of *Streptococcus pneumoniae* capsular polysaccharides which are more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant (for example, conjugates of serotype 4; serotype 6B; serotype 18C; serotype 19F; or serotype 23F).

For the purposes of this invention, the term "substantially devoid of aluminium-based adjuvants" describes a composition which does not contain sufficient aluminium-based adjuvant (for example aluminium hydroxide, and, particularly, aluminium phosphate) to cause any decrease in the immunogenicity of a pneumococcal polysaccharide conjugate of the invention in comparison to an equivalent composition comprising 3D-MPL with no added aluminium-based adjuvant. Quantitities of aluminium-based adjuvant added per dose should preferably be less than 50 μg, more preferably less than 30 μg, still more preferably less than 10 μg, and most preferably there is no aluminium-based adjuvant added to the antigenic compositions of the invention.

For the purposes of this invention, the determination of whether a pneumococcal polysaccharide conjugate is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant, this should be established as described in Example 2. As an indication of whether a composition is significantly more immunogenic when comprising 3D-MPL alone, the ratio of GMC IgG concentration (as determined in Example 2) between compositions comprising 3D-

MPL alone versus an equivalent composition comprising 3D-MPL in conjunction with aluminium phosphate adjuvant should be more than 5, preferably more than 6, more preferably more than 7, still more preferably more than 9, and most preferably more than 14.

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies, which have been designed to overcome this lack of immunogenicity, include the linking (conjugating) of the polysaccharide to large protein carriers, which provide bystander T-cell help. It is preferred that the pneumococcal polysaccharides of the invention are linked to a protein carrier which provides bystander T –cell help. Examples of these immunogenic protein carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria, Diphtheria mutant, and Tetanus toxoids (DT, CRM197 and TT respectively), Keyhole Limpet Haemocyanin (KLH), the purified protein derivative of Tuberculin (PPD), OMPC of *Neisseria meningitidis*, pneumolysin of *Streptococcus pneumoniae*, and protein D of *Haemophilus influenzae* (EP 0 594 610-B).

The present invention in a preferred embodiment provides a protein D from *Haemophilus influenzae*, or fragments thereof, as an immunogenic protein carrier for the pneumococcal polysaccharides of the invention. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragments will preferably contain the N-terminal 1/3 of the protein.

In one embodiment the antigenic composition of the invention comprises pneumococcal polysaccharide serotype (PS) 4 conjugated to an immunogenic protein and formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant. In further embodiments, the antigenic composition comprises PS 6B, 18C, 19F, or 23F, respectively, conjugated to an immunogenic protein and formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant.

In a still further embodiment of the invention, a combination antigenic composition is provided comprising two or more pneumococcal polysaccharide

conjugates from the group PS 4, PS 6B, PS 18C, PS19F, and PS 23F formulated with 3D-MPL adjuvant, where the composition is substantially devoid of aluminium-based adjuvant.

The immunogenicity of pneumococcal polysaccharide conjugates of the invention is not significantly effected by combination with other pneumococcal polysaccharide conjugates (Example 3). Accordingly, a preferred aspect of the invention provides a combination antigenic composition comprising one or more pneumococcal polysaccharide conjugates of the invention in combination with one or more further pneumococcal polysaccharide conjugates, where the composition is formulated with 3D-MPL adjuvant, but is substantially devoid of aluminium-based adjuvant.

In further preferred embodiments of the invention, combination antigenic compositions are provided which contain at least one and preferably 2, 3, 4 or all 5 of the PS 4, 6B, 18C, 19F, or 23F pneumococcal polysaccharide conjugates, and in addition any combination of other pneumococcal polysaccharide conjugates, which are formulated with 3D-MPL adjuvant but substantially devoid of aluminium-based adjuvant.

Typically the *Streptococcus pneumoniae* combination antigenic composition of the present invention will comprise polysaccharide conjugate antigens, wherein the polysaccharides are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 7 serotypes are included in the composition, for example those derived from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. More preferably still, at least 11 serotypes are included in the composition, for example the composition in one embodiment includes the capsular polysaccharide conjugates wherein the polysaccharides are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent antigenic composition above, whereas for infants serotypes 6A and 19A are advantageously included.

The antigenic compositions of the invention are preferably used as vaccine compositions to prevent (or treat) pneumococcal infections.

Further embodiments of the present invention include: the provision of the above antigenic compositions for use in medicine; a method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide conjugate, comprising the steps of administering a safe and effective amount of one of the above antigenic compositions to a patient; and the use of one of the above antigenic compositions in the manufacture of a medicament for the prevention (or treatment) of pneumococcal disease.

For the prevention/amelioration of pneumonia in the elderly (+55 years) population and Otitis media in Infants, (typically 18 months to 5 years), it is a further preferred embodiment of the invention to combine a multivalent *Streptococcus pneumonia* polysaccharide conjugate formulated as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination vaccine, include but are not limited to: pneumolysin [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC (WO 97/09994 - Briles et al); PsaA (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); Pneumococcal choline binding protein (WO 97/41151); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP 70 (WO 96/40928); M like

protein, SB patent application No. EP 0837130; and adhesin 18627, SB Patent application No. 0834568

The proteins used are preferably selected from the group pneumolysin, PsaA, PspA, CbpA (WO 97/41151) or a combination of two or more such proteins. The present invention also encompasses immunologically functional equivalents to such proteins, e.g. fragments, deletions such as transmembrane deletion variants thereof, fusions, chemically or genetically detoxified derivatives and the like, which are capable of raising substantially the same immune response as the native protein.

The antigenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said composition via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

Preferably the antigenic compositions (and vaccines) hereinbefore described are lyophilised up until they are about to be used, at which point they are extemporaneously reconstituted with diluent. More preferably they are lyophilsed in the presence of 3D-MPL, and are extemporaneously reconstituted with saline solution.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, more preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. For any proteins present in the vaccine, the protein content will typically be in the range 1-100 μg, preferably 5-50μg, most typically in the range 10 - 25μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

EXAMPLES

The examples illustrate, but do not limit the invention.

Example 1

S.pneumoniae capsular polysaccharide:

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F which were made essentially as described in EP 72513. Each polysaccharide is activated and derivatised using CDAP chemistry (WO 95/08348) and conjugated to the protein carrier. All the polysaccharides are conjugated in their native form, except for the serotype 3.Its size was reduced by micro-fluidisation.

Protein carrier:

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in *E. coli.*

EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

Starting materials

The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) Infect. Immun. 59: 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross *et al.*, 1985) in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman *et al.*, 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The *E. coli* strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil$^-$ cI857 ΔH1). N99 and SA500 are *E. coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambdaphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross *et al.*, 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambdaphage DNA integrated into the genome (Shatzman *et al.*, 1983). The chromosomal lambdaphage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH *E. coli* K12 strain N99 (F^- su^- galK2, $lacZ^-$ thr^-). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil$^-$ cI857 ΔH1). The Kil$^-$ phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil$^-$ cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson *et al.* 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37 °C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambda P_L promoter/ O_L Operator. The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from lambda P_L at low temperature by binding to O_L. Once the temperature is elevated cI is released from O_L and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D was performed as follows. The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final $OD_{650} = 60$. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

Chemistry:

Activation and coupling chemistry:

The activation and coupling conditions are specific for each polysaccharide. These are given in Table 1. Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile, CDAP (CDAP/PS ratio 0.75 mg/mg PS) was added to the polysaccharide solution. 1.5 minute later, 0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2 minutes at 25 °C. Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour. The reaction was then quenched with glycine for 30 minutes at 25 °C and overnight at 4 °C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein content of the eluted fractions was determined. The conjugates were pooled and sterile filtered on a 0.22µm sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

Characterisation:

Each conjugate was characterised and met the specifications described in Table 2. The polysaccharide content (µg/ml) was measured by the Resorcinol test and the protein content (µg/ml) by the Lowry test. The final PS/PD ratio (w/w) is determined by the ratio of the concentrations.

Residual DMAP content (ng/µg PS):

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed at SB.

Free polysaccharide content (%):

The free polysaccharide content of conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

Example 2 – Study of the Effect of Advanced Adjuvants on the Immunogenicity of the 11-Valent Pneumococcal PS-PD Conjugate Vaccine in Infant Rats

Infant rats were immunised with 11 valent pneumococcal PS-PD conjugate vaccine at a dosage of 0.1 μg each polysaccharide (made according to the method of Example 1), and using the following adjuvant formulations: none, $AlPO_4$, 3D-MPL, 3D-MPL on $AlPO_4$.

The formulation with only 3D-MPL was statistically (and surprisingly) more immunogenic (greatest GMC IgG) than for the other formulations for 5 out of 11 antigens. This was true both at high and low concentrations of 3D-MPL.

Opsonophagocytosis confirmed the GMC results.

Materials and Methods

Immunisation Protocol

Infant OFA rats were randomised to different mothers and were 7 days old when they received the first immunisation. They received 2 additional immunisations 14 and 28 days later. A bleed was performed on day 56 (28 days post III). All vaccines were injected s.c., and there were 10 rats per vaccine group.

The rats were immunised with an 11 valent pneumococcal conjugate vaccine comprising the following polysaccharide serotypes conjugated onto protein D: 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F.

Formulation

To examine the effect of different advanced adjuvants, the dosage of conjugate was held constant at 0.1 μg of each polysaccharide, and the adjuvants $AlPO_4$ and 3D-MPL were formulated in different dosages and combinations, including no adjuvant at all. These are listed numerically in Table 3 for reference.

Adsorption on AlPO4

The concentrated, adsorbed monovalents were prepared according to the following procedure. 50 μg $AlPO_4$ (pH 5.1) was mixed with 5 μg conjugated polysaccharides for 2 hours. The pH was adjusted to pH 5.1 and the mixture was left for a further 16 hours. 1500mM NaCl was added to make up the salt concentration to 150 mM. After 5 minutes 5 mg/mL 2-phenoxyethanol was added. After a further 30 minutes the pH was adjusted to 6.1, and left for more than 3 days at 4 °C.

Preparation of diluents

Three diluents were prepared in NaCl 150 mM/ 5 mg/mL phenoxyethanol

A: $AlPO_4$ at 1 mg/ml.

B: 3D-MPL on $AlPO_4$ at 250 and 1000 μg/ml respectively Weight ratio 3D-MPL/$AlPO_4$ = 5/20

C: 3D-MPL on $AlPO_4$ at 561 and 1000μg/ml respectively Weight ratio 3D-MPL/$AlPO_4$ = 50/89

Preparation of adsorbed undecavalent

The eleven concentrated, adsorbed PS-PD monovalents were mixed at the correct ratio. The complement of $AlPO_4$ was added as the diluent A. When required, 3D-MPL was added either as an aqueous solution (non adsorbed, Way 1 see below) or as the diluent B or C (3D-MPL adsorbed on $AlPO_4$ at 2 doses, Way 2, see below).

Way 1

3D-MPL was added to the combined adsorbed conjugates as an aqueous suspension. It was mixed to the undecavalent for 10 minutes at room temperature and stored at 4 °C until administration.

Way 2

3D-MPL was preadsorbed onto $AlPO_4$ before addition to the combined adsorbed conjugates (diluent B and C). To prepare 1 ml of diluent, an aqueous suspension of 3D-MPL (250 or 561 µg) was mixed with 1 mg of $AlPO_4$ in NaCl 150 mM pH 6.3 for 5 min at room temperature. This solution was diluted in NaCl pH 6.1/phenoxy and incubated overnight at 4 °C.

Preparation of non-adsorbed undecavalent

The eleven PS-PD conjugates were mixed and diluted at the right ratio in NaCl 150 mM pH 6.1, phenoxy. When required, 3D-MPL was added as a solution (non adsorbed).

The formulations for all injections were prepared 18 days before the first administration.

ELISA

The ELISA was performed to measure rat IgG using the protocol derived from the WHO Workshop on the ELISA procedure for the quantitation of IgG antibody against *Streptococcus pneumoniae* capsular polysaccharides in human serum. In essence, purified capsular polysaccharide is coated directly on the microtitre plate. Serum samples are pre-incubated with the cell-wall polysaccharide common to all pneumococcus (substance C) and which is present in ca. 0.5% in pneumococcal polysaccharides purified according to disclosure (EP 72513 B1). Jackson ImmunoLaboratories Inc. reagents were employed to detect bound murine IgG. The titration curves were referenced to internal standards (monoclonal antibodies) modeled by logistic log equation. The calculations were performed using SoftMax Pro software. The maximum absolute error on these results expected to be within a factor of 2. The relative error is less than 30%.

Opsonophagocytosis

Opsonic titres were determined for serotypes 3, 6B, 7F, 14, 19F and 23F using the CDC protocol (*Streptococcus pneumoniae* Opsonophagocytosis using Differentiated HL60 cells, version 1.1) with purified human PMN and baby rabbit complement. Modification included the use of in-house pneumococcal strains, and the phagocytic HL60 cells were replaced by purified human PMN. In addition, 3 mm glass beads were added to the microtitre wells to increase mixing, and this allowed reduction of the phagocyte:bacteria ratio which was recommended to be 400.

Results

IgG Concentrations

The geometric mean IgG concentrations determined for every serotype, and PD are shown in Tables 4 to 10. For serotypes 6B, 14, 19F and 23F, previous results obtained using a tetravalent formulation are included for comparison.

The highest IgG concentrations have been highlighted in Tables 4 to 10. The statistical p value for 3D-MPL compositions vs. 3D-MPL/ $AlPO_4$ compositions is in Table 11. Adjuvant formulation number 4 (non-adsorbed conjugates with high dose 3D-MPL) that gives the highest GMC's for 9 out of 11 cases. In 5/11 cases, MPL at the low dose is the second most immunogenic. In addition, adjuvantation gives higher GMC's than by modifying the dose for all serotypes (data not shown), and this is statistically significant for serotypes 4, 6B, 7F, 18C and 23F ($p < 0.05$ from 95% CI).

Opsonophagocytosis

Opsonophagocytosis results on pooled sera is shown for serotypes 3, 6B, 7F, 14, 19F and 23F in Tables 4 to 8. For the most part, these opsonic titres confirm the GMC IgG. Indeed, the correlation with IgG concentration is greater than 85% for serotypes 6B, 19F, 23F (data not shown). For serotype 3, it is important to note that only the 3D-MPL group induced opsonic activity above the threshold.

Conclusions

In this experiment, it was unexpected that the use of 3D-MPL alone would induce the highest IgG concentrations.

The maximal GMC IgG obtained with modifying the adjuvant was compared with the maximal GMC obtained by modifying the PS dosage, and it was found that 3D-MPL could induce significantly higher responses in 5/11 serotypes.

Table 11 shows that when 3D-MPL and 3D-MPL/ $AlPO_4$ compositions are compared (comparing the process of formulation, and the dose of 3D-MPL), 5 of the polysaccharide conjugates are significantly improved, in terms of immunogenicity, when formulated with just 3D-MPL rather than 3D-MPL plus $AlPO_4$: PS 4, PS 6B, PS 18C, PS 19F, and PS 23F.

Example 3 – Study of the effect of combination on the immunogenicity of PS 4, PS 6B, PS 18C, PS 19F, and PS 23F conjugates in adult rats

Adult rats were immunised with pneumococcal polysaccharide-protein D conjugate vaccines either individually, or combined in a multivalent composition (either tetra-, penta-, hepta-, or decavalent). Groups of 10 rats were immunised twice 28 days apart, and test bleeds were obtained on day 28 and day 42 (14 days after the 2nd dose).

The sera were tested by ELISA for IgG antibodies to the pneumococcal polysaccharides. All conjugates induced specific IgG antibodies as measured by ELISA. Table 12 shows the effect of combination of monovalent PS 6B, PS 18C, PS 19F, and PS 23F protein D conjugates on their immunogenicity in adult rats, as measured by IgG concnetration at 14 days post 2nd dose.

Statistical analysis was performed on all samples to determine if differences in antibody concentration upon combination were significant. The combination of any of serotypes PS 6B, PS 18C, PS 19F, and PS 23F protein D conjugates in a multivalent vaccine did not significantly change their immunogenicity.

Table 1
Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

Serotype	1	3 (μfluid.)	4	5	6B	7F
PS conc.(mg/ml)	2.0	3.0	2.0	7.5	5.4	3.0
PS dissolution	NaCl 2M	NaCl 2M	H_2O	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/1	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0

Serotype	9V	14	18C	19F	23F
PS conc.(mg/ml)	2.5	2.5	2.0	4.0	3.3
PS dissolution	NaCl 2M	NaCl 2M	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1	1/0.5	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	10/9.5/9.0	9.0/9.0/9.0

TABLE 2

Criteria	D01PDJ227	D03PDJ236	D4PDJ228	D5PDJ235	D6PDJ209	
Ratio PS/Prot (w/w)	1/0.66	1/1.09	1/0.86	1/0.86	1/0.69	
Free polysac. content (%) <10 %	1	1	7	9	0	
Free protein content (%) <15 %	8	<1	19	21	9	
DMAP content (ng/µg PS) < 0.5 ng/µg PS	0.2	0.6	0.4	1.2	0.3	
Molecular size (K_{av})	0.18	0.13	0.12	0.11	0.13	
Stability	no shift	no shift	no shift	low shift	no shift	
	D07PDJ225	D09PDJ222	D14PDJ202	D18PDJ221	D19PDJ206	D23PDJ212
Ratio PS/Prot (w/w)	1/0.58	1/0.80	1/0.68	1/0.62	1/0.45	1/0.74
Free polysac. content (%) <10 %	1	<1	<1	4	4	0
Free protein content (%) <15 %	8	0.3	3	21	10	12
DMAP content (ng/µg PS) <0.5 ng/µg PS	0.1	0.6	0.3	0.2	0.1	0.9
Molecular size (K_{av})	0.14	0.14	0.17	0.10	0.12	0.12
Stability	no shift	no shift	no shift	no shift	shift	no shift

Table 3. Summary Table of Adjuvant Formulations tested with 11-Valent Pneumococcal PS-PD in Infant Rats

Group	AlPO4	MPL	Method	Description
1				None
2	100			AlPO4
3		5		MPL low
4		50		MPL High
5	100	5	Way 1	Way 1 low
6	100	50	Way 1	Way 1 high
7	100	5	Way 2	Way 2 low
8	100	50	Way 2	Way 2 high

5

Table 4. Serotype 6B Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4 µg	MPL µg	Method	6B GMC IgG (µg/ml)	6B Sero-con-version	6B Opso Titre*	6B GMC IgG (µg/ml)	6B Sero-con-version	6B Opso Titre*
				Tetravalent			Undecavalent		
1				0.047	2/10	12.5	0.004	1/10	<6.25
2	100			0.048	4/10	65	0.019	4/10	<6.25
3		5					1.345	10/10	43
4		**50**					**4.927**	**10/10**	**192**
5	100	5	1				0.042	7/10	<6.25
6	100	50	1				0.255	10/10	<6.25
7	100	5	2	0.033	3/10	<6.25	0.048	8/10	<6.25
8	100	50	2				0.057	8/10	<6.25

10

Table 5. Serotype 14 Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4	MPL	Method	14 GMC IgG (µg/ml)	14 Sero-con-version	14 Opsonic Titre*	14 GMC IgG (µg/ml)	14 Sero-con-version	14 Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.046	3/10	64	0.022	3/10	<6.25
2	100			0.99	10/10	88	0.237	8/10	27
3		5					0.233	10/10	41
4		50					0.676	10/10	81
5	100	5	1				0.460	9/10	67
6	100	50	1				0.477	10/10	98
7	100	5	2	0.81	10/10	49	0.165	8/10	81
8	**100**	**50**	**2**				**1.611**	**10/10**	**133**

5

Table 6. Serotype 19F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4 µg	MPL µg	Method	19F GMC IgG (µg/ml)	19F Sero-con-version	19F Opsonic Titre*	19F GMC IgG (µg/ml)	19F Sero-con-version	19F Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.04	2/10	64	0.021	2/10	<6.25
2	100			1.07	9/10	367	0.222	7/10	79
3		5					4.028	10/10	296
4		**50**					**21.411**	**10/10**	**1276**
5	100	5	1				1.649	10/10	172
6	100	50	1				2.818	10/10	208
7	100	5	2	1.09	10/10	193	0.766	10/10	323
8	100	50	2				3.539	10/10	241

10

Table 7. Serotype 23F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants (And Comparison with Tetravalent Immunisation)

Group	AlPO4 µg	MPL µg	Method	23F GMC IgG (µg/ml)	23F Sero-conversion	23F Opsonic Titre*	23F GMC IgG (µg/ml)	23F Sero-conversion	23F Opsonic Titre*
				Tetravalent			Undecavalent		
1				0.06	2/10	<6.25	0.152	3/10	<6.25
2	100			0.29	10/10	70	0.56	8/10	<6.25
3		**5**					**2.296**	**9/10**	**389**
4		**50**					**4.969**	**10/10**	**>1600**
5	100	5	1				0.462	5/10	17
6	100	50	1				0.635	8/10	54
7	100	5	2	0.38	10/10	<6.25	0.203	3/10	18
8	100	50	2				0.501	7/10	43

Table 8. Serotypes 3 and 7F Geometric Mean IgG Concentration, Seroconversion, and Mean Opsonic Titre on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AlPO4 µg	MPL µg	Method	3 GMC IgG (µg/ml)	3 Sero-conversion	3 Opsonic Titre*	7F GMC IgG (µg/ml)	7F Sero-conversion	7F Opsonic Titre*
1				0.003	1/10	<6.25	0.040	7/10	<6.25
2	100			0.008	6/10	<6.25	0.25	9/10	43
3		**5**		**0.070**	**10/10**	**<6.25**	**2.435**	**10/10**	**477**
4		**50**		**0.108**	**10/10**	**18**	**2.569**	**10/10**	**332**
5	100	5	1	0.015	10/10	<6.25	0.579	10/10	54
6	100	50	1	0.027	10/10	<6.25	0.611	9/10	59
7	100	5	2	0.006	10/10	<6.25	0.154	8/10	30
8	100	50	2	0.034	10/10	<6.25	0.638	9/10	140

Table 9. Serotypes 1, 4 and 5 Geometric Mean IgG Concentration and Seroconversion on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AIPO4 µg	MPL µg	Method	1 GMC IgG (µg/ml)	1 Sero-con-version	4 GMC IgG (µg/ml)	4 Sero-con-version	5 GMC IgG (µg/ml)	5 Sero-con-version
1				0.026	4/10	0.005	0/10	0.040	3/10
2	100			0.282	8/10	0.052	5/10	0.774	9/10
3		5		1.614	10/10	**3.452**	**10/10**	7.927	10/10
4		**50**		2.261	10/10	**7.102**	**10/10**	**13.974**	**10/10**
5	100	5	1	0.568	10/10	0.676	10/10	3.015	10/10
6	100	50	1	1.430	10/10	0.419	9/10	5.755	10/10
7	100	5	2	0.478	10/10	0.267	9/10	2.062	10/10
8	100	50	2	1.458	10/10	0.423	10/10	5.009	10/10

Table 10. Serotypes 9V, 18C and PD Geometric Mean IgG Concentration and Seroconversion on Day 28 Post III Immunisation of Infant Rats with 11-Valent PS-PD using Different Adjuvants

Group	AIPO4 µg	MPL µg	Method	9V GMC IgG (µg/ml)	9V Sero-con-version	18C GMC IgG (µg/ml)	18C Sero-con-version	PD GMC IgG (µg/ml)	PD Sero-con-version
1				0.018	0/10	0.013	1/10	0.003	0/10
2	100			0.489	6/10	0.092	5/10	0.993	10/10
3		5		0.482	7/10	**6.560**	**10/10**	3.349	10/10
4		**50**		**11.421**	**10/10**	**14.023**	10/10	5.446	10/10
5	100	5	1	2.133	9/10	0.690	10/10	**11.407**	**10/10**
6	100	50	1	2.558	10/10	1.771	10/10	1.258	10/10
7	100	5	2	1.536	10/10	0.528	10/10	1.665	8/10
8	100	50	2	2.448	9/10	0.980	10/10	5.665	10/10

Table 11: The statistical significance (p value) of whether certain pneumococcal polysaccharide conjugates had improved immunogenicty when formulated with 3D-MPL alone versus with 3D-MPL/AlPO4. A p value under 0.01 is considered highly significant. Way 1 and Way 2 indicate the method of formulation.

serotype	50 µg 3D-MPL v 3D-MPL/$AlPO_4$		5 µg 3D-MPL vs 3D-MPL/$AlPO_4$	
	Way 1	Way 2	Way 1	Way 2
1	0.3	0.05	0.079	0.11
3	0.075	0.01	0.27	0.008
4	0.002	0.0003	0.02	0.003
5	0.04	0.002	0.1	0.12
6B	0.001	0.0001	0.001	0.0006
7F	0.13	0.15	0.01	0.005
9V	0.02	0.02	0.1	0.04
14	0.65	0.21	0.3	0.66
18C	0.0008	0.0002	0.006	0.004
19F	0.0009	0.006	0.21	0.04
23F	0.002	0.0004	0.01	0.0004

Table 12: Geometric Mean IgG concentration (µg/mL) on day 14 post 2nd dose after immunisation of adult rats with 1.0 µg polysaccharide-protein D conjugate alone or combined in tetravalent, pentavalent, heptavalent or decavalent vaccine. These data are combined from 5 separate experiments.

Serotypes Vaccines	4 H	6B T	18C H	19F T	23F T
Alone	9.3	0.11	15	5.2	2.5
Combined	4	0.23	3.7	3.7	2.8
T: combined in tetravalent (T) (PS 6B, 14, 19F, 23F), pentavalent (T plus PS 3), heptavalent (H) (T plus PS 4, 9V and 18C), and decavalent (H plus PS 1, 5 and 7F) combination vaccines. H: combined in heptavalent (H) (T plus PS 4, 9V and 18C), and decavalent (H plus PS 1, 5 and 7F) combination vaccines.					

We claim:

1. An antigenic composition comprising one or more *Streptococcus pneumoniae* capsular polysaccharide conjugates adjuvanted with 3D-MPL and substantially devoid of aluminium-based adjuvants, wherein at least one of the *Streptococcus pneumoniae* polysaccharide conjugates is significantly more immunogenic in compositions comprising 3D-MPL in comparison with compositions comprising 3D-MPL in conjunction with an aluminium-based adjuvant.

2. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 4 conjugated to an immunogenic protein.

3. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 6B conjugated to an immunogenic protein.

4. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 18C conjugated to an immunogenic protein.

5. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 19F conjugated to an immunogenic protein.

6. The antigenic composition of claim 1 comprising the *Streptococcus pneumoniae* capsular polysaccharide serotype 23F conjugated to an immunogenic protein.

7. A combination antigenic composition substantially devoid of aluminium-based adjuvants and comprising 3D-MPL adjuvant and two or more pneumococcal polysaccharide conjugates chosen from the group consisting of:

serotype 4;

serotype 6B;

serotype 18C;

serotype 19F; and
serotype 23F.

8. The antigenic compositions of claims 1-7 wherein the *Streptococcus pneumoniae* capsular polysaccharides are conjugated to a protein chosen from the group consisting of:

tetanus toxoid;
OMPC of *Neisseria meningitidis*;
diphtheria toxoid;
pneumolysin of *Streptococcus pneumoniae*; or
CRM197.

9. The antigenic compositions of claims 1-7 wherein the *Streptococcus pneumoniae* capsular polysaccharides are conjugated to a protein D of *Haemophilus influenzae*.

10. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 6B, 14, 19F and 23F.

11. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 4, 6B, 9V, 14, 18C, 19F and 23F.

12. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F.

13. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6B, 7F, 8, 9V, 12F, 14, 18C, 19F and 23F.

14. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F.

15. The combination antigenic composition of claim 7 containing conjugates of *Streptococcus pneumoniae* capsular polysaccharide serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F.

16. A lyophilsed antigenic composition according to claims 1-15.

17. The antigenic composition of claims 1-16, which is a vaccine composition.

18. A method of inducing an immune response to a *Streptococcus pneumoniae* capsular polysaccharide conjugate, said method comprising administering a safe and effective amount of an antigenic composition as claimed herein to a patient.

19. Use of an antigenic composition as claimed herein in the manufacture of a medicament for the prevention of pneumococcal disease.

4

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Rule 16 of the Patents Rules 1990 is the main rule governing the completion and filing of this form.

❶ Title of invention

1 Please give the title of the invention: Vaccines

Do not give trading styles, for example, 'Trading as XYZ company', nationality or former names, for example, 'formerly (known as) ABC Ltd' as these are not required.

❷ Applicant's details

☐ **First or only applicant**

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Agent's address SmithKline Beecham
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VACCINES

The present invention relates to polysaccharide antigen vaccines, their manufacture and the use of such polysaccharides in medicines. In particular the present invention relates to combined vaccines comprising a polysaccharide antigen, typically a polysaccharide conjugate antigen optionally formulated with either or both a Th1 inducing adjuvant and a protein antigen from Streptococcus Pneumoniae. Such vaccines are particularly useful in the protection of the elderly from Pneumonia.

Streptococcus pneumoniae is a Gram-positive bacteria that is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

Polysaccharide antigen based vaccines are well known in the art. Four that have been licensed for human use include the Vi polysaccharide of *Salmonella typhi*, the PRP polysaccharide from *Haemophilus influenzae*, the tetravalent meningococcal vaccine composed of serotypes A, C, W135 and Y, and the 23-Valent pneumococcal vaccine composed of the polysaccharides corresponding to serotypes 1, 2, 3,4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33.

The latter three vaccines confer protection against bacteria causing respiratory infections resulting in severe morbidity and mortality in infants, yet

these vaccines have not been licensed for use in children less than two years of age because they are poorly immunogenic in this age group.

The 23-valent pneumococcal vaccine has shown a wide variation in clinical efficacy, from 0% to 81% (Fedson et al. Arch Intern Med. 154: 2531-2535). The efficacy appears to be related to the risk group that is being immunised, such as the elderly, Hodgkin's disease, splenectomy, sickle cell disease and agammaglobulinemics (Fine et al Arch Intern Med. 154:2666-2677), and also to the disease manifestation. Pneumococcal pneumonia and Otitis media are diseases, which do not have demonstrated protection by the 23-valent vaccine. It is generally accepted that the protective efficacy of the pneumococcal vaccine is more or less related to the concentration of antibody induced upon vaccination; indeed, the 23 polysaccharides were accepted for licensure solely upon the immunogenicity of each component polysaccharide (Ed. Williams et al. New York Academy of Sciences 1995 pp. 241-249).

The present invention provides an improved vaccine particularly for the prevention or amelioration of Pnemococcal infection of the elderly and/or infants.

In the context of the invention a patient is considered elderly if they are 55 years or over in age, typically over 60 years and more generally over 65years. Thus in one embodiment of the invention there is provided a vaccine composition comprising a polysaccharide antigen and a Th1 adjuvant for the prevention of pneumonia in the elderly.

In a second embodiment, the present invention provides a vaccine composition, suitable for use in the elderly and/or Infants comprising at least one *Streptococcus pneumoniae* polysaccharide antigen and one *Streptococcus pneumoniae* protein antigen.

In a third embodiment there is provided a vaccine comprising at least one *Streptococcus pneumoniae* polysaccharide antigen and one *Streptococcus pneumoniae* protein antigen and a Th1 adjuvant.

Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise polysaccharide conjugate antigens, wherein the

polysaccharide are derived from at least four serotypes. Preferably the four serotypes include 6B, 14, 19F and 23F. More preferably, at least 11 serotypes are included in the vaccine, for example the vaccine in one embodiment includes the capsular polysaccharide conjugate wherein the polysaccharide are derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are included. In a preferred embodiment of the invention at least 13 polysaccharide conjugates are included, although more valents, for example 23 valents (such as included in the currently licensed vaccine) are contemplated by the invention.

For elderly vaccination (for the prevention of pneumonia) it is advantageous to include serotypes 8 and 12F to the 11 valent vaccine above, whereas for infants serotypes 6A and 19A are advantageously included.

For the prevention/amelioration of pneumoniae in the elderly (+55 years) population and Otitis media in Infants, (typically 18 months to 5 years), it is a preferred embodiment of the invention to combine a multivalent streptococcus pneumonia polysaccharide as herein described with a *Streptococcus pneumoniae* protein or immunologically functional equivalent thereof. Preferred proteins to be included in such a combination, include but are not limited to, pneumolysin (Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2." Mitchell TJ, Mendez F, Paton JC, Andrew PW, Boulnois GJ, Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ. WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al) -.WO 99/03884 (NAVA)), PspA and transmembrane deletion variants thereof US 5804193 (Briles et al), PspC (WO 97/09994 - Briles et al). PsaA (Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*." Berry AM, Paton JC). Streptococcal choline binding protein (WO97/41151); Glyceraldehyde – 3- phosphate – dehydrogenase (I&I 64: 3544), HSP 70 (WO96/40928), M like

protein SB patent application No EP 0837130 and adhesin 18627 SB Patent application No. 0834568

The proteins used in the present invention are preferably selected from the group pneumolysin, PsaA, PspA, CbpA (WO97/41151) or a combination of two or more such proteins. The present invention also encompasses immunologically functional equivalents to such proteins, e.g. fragments, deletions such as transmembrane deletion variants thereof, fusions, chemically or genetically detoxified derivatives and the like which are capable of raising substantially the same immune response as the native protein.

Accordingly in an embodiment of the invention there is provided a Streptococcus pneumoniae vaccine comprising a polysaccharide conjugate vaccine comprising polysaccharide antigens derived from at least four serotypes, preferably at least seven serotypes, more preferably at least eleven serotypes and at least one, but preferably two Streptococcus pneumoniae proteins. Preferably one of the proteins is Pneumolysin or PsaA or PspA or CbpA. A preferred combination contains at least Pneumolysin or a derivative thereof and Psp A.

Amongst the problems associated with the polysaccharide approach to vaccination, is the fact that polysaccharides *per se* are poor immunogens. Strategies, which have been designed to overcome this lack of immunogenicity, include the linking of the polysaccharide to large protein carriers, which provide bystander T-cell help. It is preferred that the polysaccharides utilised in the invention are linked to a protein carrier which provide bystander T –cell help. Examples of these highly carriers which are currently commonly used for the production of polysaccharide immunogens include the Diphtheria and Tetanus toxoids (DT, DT crm197 and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD).

A number of problems are, however, associated with each of these commonly used carriers, including both problems in production of GMP constructs and also in immunological characteristics of the constructs. The present invention provides in a preferred embodiment a new carrier for use in the preparation of

polysaccharide -based immunogen constructs, that does not suffer from these disadvantages.

Despite the common use of these carriers and their success in the induction of anti polysaccharide antibody responses they are associated with several drawbacks. For example, it is known that antigen specific immune responses may be suppressed by the presence of pre-existing antibodies directed against the carrier, in this case Tetanus toxin (Di John *et al*; Lancet, December 16, 1989). In the population at large, a very high percentage of people will have pre-existing immunity to both DT and TT as people are routinely vaccinated with these antigens. In the UK for example 95% of children receive the DTP vaccine comprising both DT and TT. Other authors have described the problem of epitope suppression to peptide vaccines in animal models (Sad *et al*, Immunology, 1991; 74:223-227; Schutze *et al*, J. Immunol. 135: 4, 1985; 2319-2322).

In addition, for vaccine which require regular boosting the use of highly immunogenic carriers such as TT and DT are likely to suppress the polysaccharide antibody response after several injections. These multiple vaccinations may also be accompanied by undesirable reactions such as delayed type hyperresponsiveness (DTH).

KLH is known as potent immunogen and has already been used as a carrier for IgE peptides in human clinical trials. However, some adverse reactions (DTH-like reactions or IgE sensitisation) as well as antibody responses against antibody have been observed.

The selection of a carrier protein, therefore, for a polysaccharide based vaccine requires a balance between the necessity to use a carrier working in all patients (broad MHC recognition) and the induction of high levels of anti-polysaccharide antibody responses and of low antibody response against the carrier.

The carriers used previously for polysaccharide based vaccines, therefore have many disadvantages.

The present invention therefore in a preferred embodiment provides a protein D from *Haemophilus influenzae*, or fragments thereof, as a carrier for

polysaccharide based immunogenic composition, such as vaccines. Fragments suitable for use include fragments encompassing T-helper epitopes. In particular protein D fragment will preferably contain the N-terminal 1/3 of the protein.

Protein D is an IgD-binding protein from *Haemophilus influenzae* (EP 0 594 610 B1) and is a potential immunogen.

The vaccines of the present invention are preferably adjuvanted. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

It is preferred that the adjuvant be selected to be a preferential inducer of a TH1 type of response to enable a cell mediated response to be generated. High levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of Il-4, IL-5, IL-6, IL-10. Suitable adjuvant systems include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt .

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

Unmethylated CpG containing oligonucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

The vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts.

In one embodiment there is a method of preventing or ameliorating Pneumoniae in an elderly human comprising administering a safe and effective amount of a vaccine, as described herein, comprising a Streptoccocus Pneumoniae polysaccharide antigen and a Th1 adjuvant, optionally with a streptoccocus pneumoniae protein, to said elderly patient.

In a further embodiment there is provided a method of preventing or ameliorating Otitis media in Infants, comprising administering a safe and effective

amount of a vaccine comprising a Streptococcus Pneumoniae polysaccharide antigen and a Streptococcus pneumoniae protein antigen optionally with a Th1 adjuvant, to said Infant.

Preferably in the methods of the invention as descibed above the polysaccharide antigen is presented as polysaccharide protein conjugate.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, preferably 0.1-50 μg, preferably 0.1-10 μg, of which 1 to 5 μg is the most preferable range. The protein content of the vaccine will typically be in the range1 -100μg, preferably 5-50μg, most typically in the range 10 - 25μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

EXAMPLE I

S.pneumoniae capsular polysaccharide:

The 11-valent candidate vaccine includes the capsular polysaccharides serotypes 1,3,4,5,6B, 7F, 9V,14,18C,19F and 23F which were made essentially as described in EP72513.

Each polysaccharide is activated and derivatised using the CDAP chemistry (WO/95/08348) and conjugated to the protein carrier.

All the polysaccharides are conjugated in their native form, except for the serotype 3.Its size was reduced by micro-fluidisation.

Protein carrier:

The protein carrier selected is the recombinant protein D (PD) from Non typeable *Haemophilus influenzae*, expressed in *E.coli*.

EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

Starting materials

The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) I & I *59* : 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross et al, 1985) in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman et al., 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The E. coli strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil - cI857 ΔH1). N99 and SA500 are E. coli K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambdaphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross

et al., 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambdaphage DNA integrated into the genome (Shatzman et al., 1983). The chromosomal lambdaphage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH E. coli K12 strain N99 (F - su - galK2, lacZ - thr -). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil - cI857 ?H1). The Kil -phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil - cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson et al. 1991) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between

NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labeled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----

NS1 Protein D

The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37°C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambdaP_L promoter/ O_L Operator.

The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from lambdaP_L at low temperature by binding to O_L. Once the

temperature is elevated cI is released from O_L and protein D is expressed. At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D is described below:

The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final OD_{650} = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris are removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 μm membrane.

Chemistry:

Activation and coupling chemistry:

The activation and coupling conditions are specific for each polysaccharide .These are given in Table 1.

Native polysaccharide (except for PS3) was dissolved in NaCl 2M or in water for injection. The optimal polysaccharide concentration was evaluated for all the serotypes.

From a 100 mg/ml stock solution in acetonitrile ,CDAP (CDAP/PS ratio:0.75 mg/mg PS) was added to the polysaccharide solution.1.5 minute later,0.2M triethylamine was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 2minutes at 25°C.Protein D (the quantity depends on the initial PS/PD ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for 1 hour.

Then, the reaction was quenched with glycine for 30 minutes at 25°C and overnight at 4°C.

The conjugates were purified by gel filtration using a Sephacryl 500HR gel filtration column equilibrated with 0.2M NaCl.

The carbohydrate and protein contents of the eluted fractions were determined .The conjugates were pooled and sterile filtered on a 0.22μm sterilizing membrane. The PS/Protein ratios in the conjugate preparations were determined.

Characterisation:

Each conjugate was characterised and met the specifications described in Table 2.

Polysaccharide and protein content (μg/ml):

The polysaccharide content was measured by the Resorcinol test and the protein content by the Lowry test. The final PS/PD ratio(w/w) is determined by the ratio of the concentrations.

Residual DMAP content (ng/μg PS):

The activation of the polysaccharide with CDAP introduces a cyanate group in the polysaccharide and DMAP (4-dimethylamino-pyridin) is liberated. The residual DMAP content was determined by a specific assay developed and validated at SB.

Free polysaccharide content (%):

The free polysaccharide content on conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-PD antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant . The absence of conjugate was also controlled by an α-PD/α-PS ELISA.

Example 2: Pneumolysin was obtained according to the methods of Biochim Biophys Acta 1989 Jan 23;1007(1):67-72 "Expression of the pneumolysin gene in Escherichia coli: rapid purification and biological properties." Mitchell TJ, Walker JA, Saunders FK, Andrew PW, Boulnois GJ.

PspA and transmembrane deletion variants thereof can be obtained according to the methods of US 5804193 (Briles et al).

CbpA can be prepared according to the methods of WO 97/41151.

Adjuvant compositions

Protein, either individually or together, from the above examples maybe formulated with the undecavalent Streptoccocus polysaccharide combination of example 1 and as adjuvant, the formulation may comprise a mixture of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and aluminium hydroxide, or of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and aluminium phosphate, or 3D-MPL

and/or QS21 optionally in an oil/water emulsion, and optionally formulated with cholesterol, or aluminium salt alone, preferably aluminium phosphate.

3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria *Salmonella minnesota.*

Experiments performed at SmithKline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral and a TH1 type of cellular immunity.

QS21: is one saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it activates both antigen-specific lymphoproliferation and CTLs to several antigens.

Vaccine containing an antigen of the invention containing 3D-MPL and alum may be prepared in analogous manner to that described in WO93/19780 or 92/16231.

Experiments performed at SmithKline Beecham Biologicals have demonstrated a clear synergistic effect of combinations of 3D-MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses. Vaccines containing an antigen such antigens are described in US 5750110.

The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5% tocopherol 0.4% Tween 80 and had an average particle size of 180 nm and is known as SB62 (see WO 95/17210).

Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to MPL/QS21 further increases their immunostimulant properties.

Preparation of emulsion SB62 (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S

microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

Table 1

Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D conjugates

Serotype	1	3 (μfluid.)	4	5	6B	7F
PS conc.(mg/ml)	2.0	3.0	2.0	7.5	5.4	3.0
PS dissolution	NaCl 2M	NaCl 2M	H_2O	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/1	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0

Serotype	9V	14	18C	19F	23F
PS conc.(mg/ml)	2.5	2.5	2.0	4.0	3.3
PS dissolution	NaCl 2M	NaCl 2M	H_2O	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1	1/0.5	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a = pH_c = pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	10/9.5/9.0	9.0/9.0/9.0

TABLE 2

Criteria	D01PDJ227	D03PDJ236	D4PDJ228	D5PDJ235	D6PDJ209	
Ratio PS/Prot (w/w)	1/0.66	1/1.09	1/0.86	1/0.86	1/0.69	
Free polysac. content (%) <10 %	1	1	7	9	0	
Free protein content (%) <15 %	8	<1	19	21	9	
DMAP content (ng/μg PS) < 0.5 ng/μg PS	0.2	0.6	0.4	1.2	0.3	
Molecular size (K_{av})	0.18	0.13	0.12	0.11	0.13	
Stability	no shift	no shift	no shift	low shift	no shift	
	D07PDJ225	D09PDJ222	D14PDJ202	D18PDJ221	D19PDJ206	D23PDJ212
Ratio PS/Prot (w/w)	1/0.58	1/0.80	1/0.68	1/0.62	1/0.45	1/0.74
Free polysac. content (%) <10 %	1	<1	<1	4	4	0
Free protein content (%) <15 %	8	0.3	3	21	10	12
DMAP content (ng/μg PS) <0.5 ng/μg PS	0.1	0.6	0.3	0.2	0.1	0.9
Molecular size (K_{av})	0.14	0.14	0.17	0.10	0.12	0.12
Stability	no shift	no shift	no shift	no shift	shift	no shift

Claims:

1. A vaccine composition, comprising at least one Streptococcus pneumoniae polysaccharide antigen and one Streptococcus pneumoniae protein antigen or immunologically functional equivalent thereof.

2 A vaccine as claimed in claim 1 wherin the polysaccharide antigen is presented in the form of a polysaccharide–protein carrier conjugate.

3 A vaccine as claimed in claim 2 wherein the carrier protein is selected from the group Diphtheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD).

4 A vaccine as claimed in any of claim 1 to 3 wherein the vaccine comprises at least four polysaccharide antigens from different serotypes.

5 A vaccine as claimed in claim 4 wherein the vaccine comprises polysaccharides from serotypes 6B, 14, 19F and 23F.

6 A vaccine as claimed herein wherein the vaccine comprises at least eleven polysaccharide antigens from different serotypes.

7 A vaccine as claimed herein wherein the vaccine comprises at least thirteen polysaccharide antigens from different serotypes.

8 A vaccine as claimed herein wherein the streptococcus pneumoniae protein or immunologically functional equivalent thereof is selected from the group: pneumolysin , PspA and transmembrane deletion variants thereof, PspC , PsaA , Streptococcal choline binding protein, Glyceraldehyde –3- phosphate – dehydrogenase and CbpA.

9 A vaccine as claimed herein comprising at least two Streptococcus pneumoniae proteins or immunologically functional equivalents thereof.

10 A vaccine as claimed herein additionally comprising an adjuvant.

11 A vaccine as claimed in claim 10, wherein the adjuvant comprises an aluminium salt.

12 A vaccine as claimed in claim 10, wherein the adjuvant is a preferential inducer of a TH1 response.

13 A vaccine as claimed in claim 10 or 12, wherein the adjuvant comprises at least one of the following: Monophosphoryl Lipid A or derivative thereof, a saponin immunostimulant, an immunostimulatory CpG oligonucleotide.

14 A vaccine as claimed in claim 13 wherein the adjuvant comprises a carrier selected from the group comprising an oil in water emulsion, liposome and aluminium salt.

15 A vaccine composition as claimed herein for use as a medicament.

16 A method of preventing or ameliorating Streptoccocus Pneumoniae infection in a patient over 55 years, comprising adminstering an effective amount of a vaccine comprising a streptococcus Pneumoniae polysaccharide and either a TH1 inducing adjuvant or a Streptococcus Pneumoniae protein or both.

17 Use of a Streptoccocus pneumoniae protein antigen and/or a Th1 inducing adjuvant and polysaccharide antigen in combination, in the manufacture of a medicament for the prevention of pneumonia in patients over 55 years.

18 A method of making a vaccine as claimed herein, comprising the step of mixing the polysaccharide antigen and the protein antigen.

19. A method of preventing or ameliorating Otitis media in Infants, comprising administering a safe and effective amount of a vaccine comprising a Streptococcus Pneumoniae polysaccharide antigen and a Streptococcus pneumoniae protein antigen optionally with a Th1 adjuvant, to said Infant.